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INVOLVEMENT OF DNA REPLICATION AND REPAIR IN MUTAGENESIS OF *HAEMOPHILUS INFLUENZAE* INDUCED BY *N*-NITROSOCARBARYL

A Dissertation

Presented for the

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Kenneth Loren Beattie

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ABSTRACT

An investigation of the cellular processes involved in mutagenesis was conducted, using the bacterium Haemophilus influenzae treated with the alkylating agent N-nitrosocarbaryl (NC). A temperature-sensitive DNA synthesis mutant (strain dna9) was treated with NC, then incubated at the permissive (36° C) and nonpermissive (41° C) temperatures. At various times lysates were made and used to transform a second culture to mutant phenotype (novobiocin resistance). It is known that premutational lesions present in the DNA immediately after treatment with mutagen do not produce an appreciable number of mutations in the second culture through transformation. However, if the treated cells are washed and allowed to grow for a time before the DNA is extracted, the mutation yield obtained through transformation approaches that obtained when the same cells are grown for several generations and tested directly for mutation frequency. This increase in the ability of DNA from lysed cells to produce mutants through transformation, known as "mutation fixation", represents conversion of premutational lesions to a form that can be detected by transformation, perhaps the ultimate change in the nucleotide sequence in the DNA.

At the permissive temperature mutation fixation continued at an approximately linear rate during nearly half of the first round of DNA replication after treatment with NC. In the absence of DNA replication (41° C), most but not all of the mutation fixation was eliminated. The data suggest that premutational lesions occur over the entire chromosome

and indicate that the bulk of the mutation fixation requires DNA replication, but that a process independent of replication, probably an error-prone repair system, also is responsible for part of the mutation fixation.

When strain dna9 was treated with NC and then incubated at 41° C for a time (stopping DNA replication and the bulk of the mutation fixation) before being grown at 36° C, a large decrease in the final mutation frequency was seen. This suggests that a repair mechanism still functional in the absence of DNA replication is capable of removing premutational lesions from the H. influenzae DNA.

Repair of premutational lesions and nonreplicative mutation fixation were observed in three different strains carrying mutations affecting excision-repair of UV-induced pyrimidine dimers, indicating that the pyrimidine dimer-excising mechanism of H. influenzae is not involved in NC-induced mutagenesis.

The recombination-deficient mutant rec1 is apparently deficient in the replication-dependent mode of mutation fixation. This conclusion is based on the following results:

1. NC-induced mutagenesis is lower in the rec1 strain than in rec$^+$ cells.
2. Repair of premutational lesions (which depends on the existence of replication-dependent mutation fixation for its detection) was not detected in the rec1 strain.
3. When nonreplicative mutation fixation and final mutation frequency were measured in the same experiment about 1/4 to 1/3 of the final mutation yield could be accounted for

by nonreplicative mutation fixation in the rec$^+$ strain, whereas all of the mutation could be accounted for in the recl strain by the nonreplicative mutation fixation.

4. When mutation fixation in strain dna9 recl was followed at the permissive (36° C) and nonpermissive (41° C) temperatures it became apparent that in the recl strain replication-dependent mutation fixation occurs at early times but these newly fixed mutations are unstable and disappear at later times, leaving only the mutations fixed by the nonreplicative process.

The recl strain exhibits normal repair of NC-induced single strand breaks or alkali labile bonds in the DNA labeled before treatment, but is slow in joining discontinuities present in DNA synthesized after treatment. Compared to strain dna9, treatment of strain dna9 recl with NC results in excessive degradation of DNA labeled either before or after treatment occurs, accompanied by increased lethality.

The results obtained do not indicate whether in NC-treated H. influenzae cells the replication-dependent mode of mutation fixation occurs by direct mispairing opposite certain alkylation-induced lesions or by error-prone joining of interruptions present in the DNA synthesized after treatment.

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I. INTRODUCTION

Mutation: A Cellular Process

Mutation, a heritable change in the nucleotide sequence in the DNA of a cell, can be induced by exposure to certain chemicals or radiation. It can also occur spontaneously at a lower frequency. In the case of induced mutation a large effort has been directed toward characterizing the initial reactions between mutagenic agents and DNA as well as the ultimate changes in the nucleotide sequence. Between the initial reaction and the final sequence alteration lie cellular events that are still poorly understood. Much of the evidence that has been obtained concerning the cellular processes involved in mutagenesis appears in several recent reviews and symposia[8, 16, 31, 145, 149]. Much of this material will be discussed in section IV. This dissertation describes work with the bacterium Haemophilus influenzae that has begun to identify the cellular processes that lead to mutation after treatment with one class of alkylating agents, N-nitroso compounds.

The Haemophilus influenzae System

The usefulness of the H. influenzae system lies in the following facts:

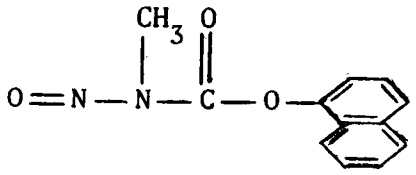
1. The presence of mutations in isolated DNA can be assayed by transformation of a competent culture to mutant phenotype.
2. When H. influenzae cells are treated with N-nitroso compounds, then washed to stop the reaction and lysed immediately, the

extracted DNA produces very few mutations in the transformation assay[57].

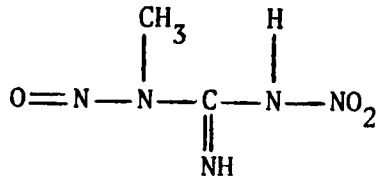
3. If the treated cells are washed and allowed to grow for a time before the DNA is extracted, the mutation yield obtained through transformation approaches that obtained when the same cells are grown for several generations and tested directly for mutation frequency[57].

Thus, the premutational mutagen-induced lesions present immediately after treatment of the cells apparently cannot produce an appreciable number of mutations in the recipient genome via transformation. The reason for this is not known, but premutational lesions may inhibit integration or may be efficiently repaired in the recipient cell. However, after cellular processes are allowed to act on the DNA containing the lesions, a later stage in the mutational process is reached, which is reflected by the ability of the extracted DNA to produce mutants in a second culture via transformation. This later stage may represent a heritable change in the nucleotide sequence in the DNA. The term "mutation fixation" has been chosen to describe the increase with time in the ability of lysates made from mutagen-treated cells to transform a second culture to mutant phenotype[58].

All the work reported here utilized mutation to resistance to novobiocin (1 μg per ml) induced by N-nitrosocarbaryl (N-methyl-N-nitroso-1-naphthylcarbamate, NC). The structure of this N-nitroso compound, along with that of the commonly used N-nitroso compound N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) is shown below.



NC



MNNG

NC is formed by reaction between carbaryl (a widely used insecticide known by the trade name "Sevin") and nitrite under mildly acidic conditions[32]. NC is highly mutagenic like MNNG but for unknown reasons 50-100 times less NC (on a molar basis) is required for a given biological effect, making it the most potently mutagenic compound known for H. influenzae[33].

Novobiocin resistance is closely linked to streptomycin resistance on the H. influenzae chromosome. Therefore, if cells that are streptomycin-resistant and novobiocin-sensitive are treated with mutagen, and the DNA is extracted and used to transform another culture to streptomycin resistance, a large fraction of the transformants also integrate the novobiocin region, which may contain mutagen-induced alterations that can subsequently result in mutations to novobiocin resistance in the recipient culture. The procedure for measuring mutation fixation is represented in Figure 1.

Figure 1. Procedure for measuring mutation fixation.

Streptomycin-resistant, novobiocin-sensitive cells are treated with NC then incubated for various times in growth medium before being used to make cell lysates. The lysates are used to transform drug-sensitive cells to streptomycin resistance, then the transformants are plated in novobiocin agar. The frequency of novobiocin-resistant mutants among the streptomycin-resistant transformants is a measure of the extent of mutation fixation.

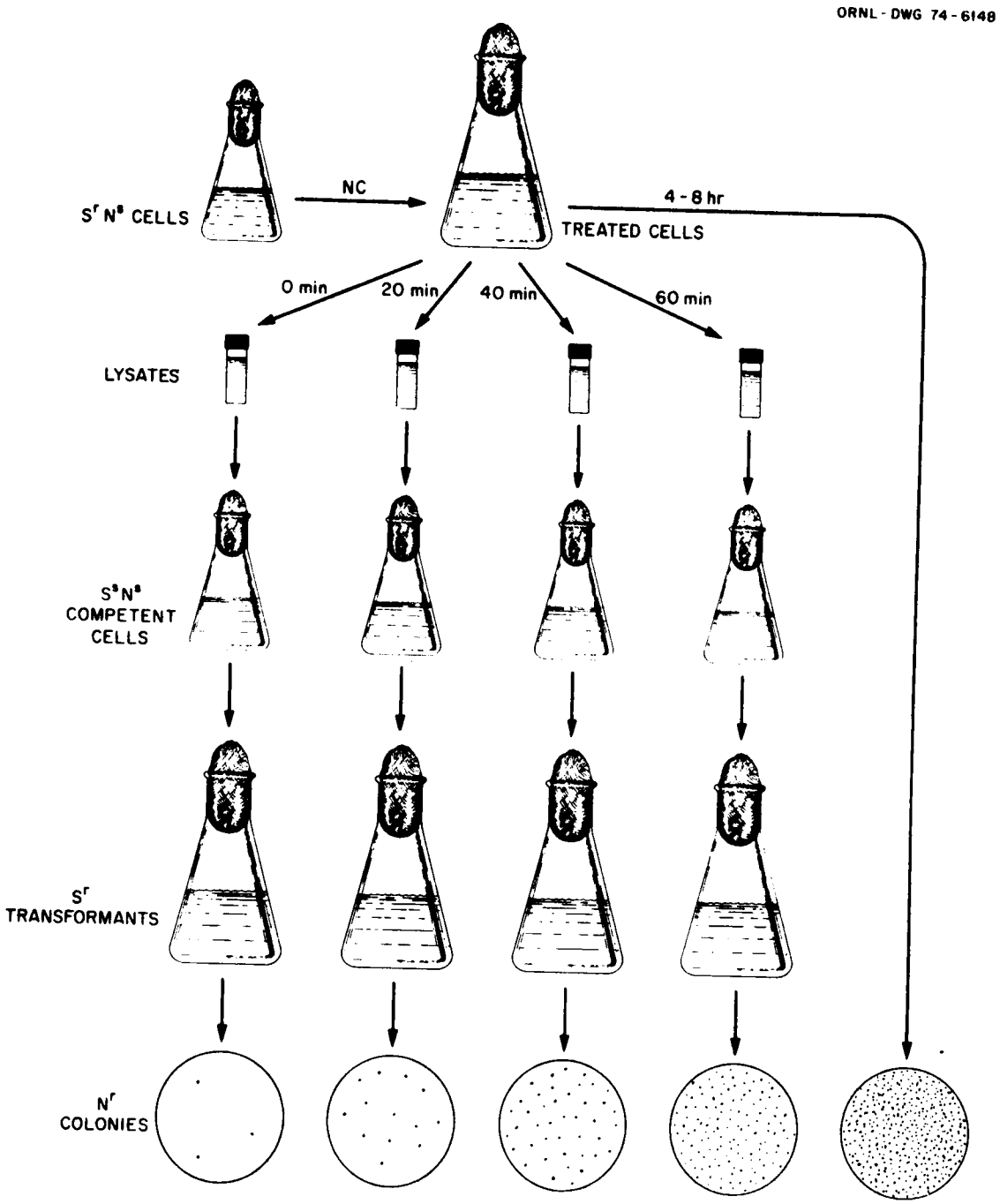

ORNL - DWG 74 - 6148
Sr N^s CELLS
NC
4 - 8 hr
TREATED CELLS
0 min
20 min
40 min
60 min
LYSATES
S^s N^s
COMPETENT
CELLS
S^r
TRANSFORMANTS
N^r
COLONIES

Figure 1

II. MATERIALS AND METHODS

Bacterial Strains

The wild-type Haemophilus influenzae strain Rd, strain BC200 which lacks an inducible defective prophage[3, 6], the ultraviolet light-sensitive strains DB112, DB115 and DB116, and the recombination-deficient, ultraviolet light-sensitive strain DB117 were obtained from Dr. J. K. Setlow. Properties of the latter four strains have been previously described[111, 113, 114].

The temperature-sensitive DNA synthesis mutant dna9 was isolated as follows. A thymidine-requiring mutant (spontaneous mutant isolated from strain Rd by the method of Chevallier and Greth[22]) previously treated with MNNG (10% survival) was grown at 34° C to exponential phase in supplemented Brain Heart Infusion (BHI) (see below), then incubated at 42° C for 20 minutes (half a generation). Then a large excess (100 μg per ml) of dBrUrd was added, and the culture was incubated at 42° C for 20 minutes longer, then irradiated in buffer (see below) with 10^4 ergs per mm^2 313-nm light to selectively kill the wild-type cells, which had incorporated dBrUrd into their DNA. The survivors were grown to stationary phase at 34° C. After six more cycles of 42° C, dBrUrd, and 313-nm light treatment, essentially no wild-type cells remained in the culture. Single-colony isolates were made and tested for uptake of (^{3}H)dThd at 34° and at 40° C. The dna9 mutation, which causes almost immediate cessation of uptake of (^{3}H)dThd when the temperature is raised from 37° to 40° C, was moved into the wild-type strain Rd by J. E. LeClerc by transformation, followed by replica plating at 37° and 40° C.

The dna9 strain used in these studies was made resistant to 250 μg per ml streptomycin by transformation with DNA from a streptomycin-resistant strain.

The dna9 rec1 double mutant, obtained from J. E. LeClerc, was constructed by transformation of strain dna9 with DNA from the UV-sensitive strain DB117, carrying the rec1 mutation. A UV-sensitive, recombination-deficient transformant was isolated by replica plating with UV doses at 254 nm of 0 and 300 ergs per mm^2.

The dna9 uvr1 double mutant was constructed as follows. Streptomycin-sensitive strain dna9 was transformed with DNA from streptomycin-resistant strain DB112 (which carries the uvr1 mutation, linked to the streptomycin locus) and 617 streptomycin-resistant transformant clones were individually tested for UV sensitivity by the following spot test. Clones were grown to near stationary phase in BHI broth, then spotted onto the surface of BHI agar (see below), dried at 37° C and irradiated with 100 ergs per mm^2 254-nm light. Two UV-sensitive clones were obtained. Uptake of (^{3}H)dThd at 37° and 41° C revealed that only one of these clones still carried the dna9 mutation. A UV survival curve indicated that this strain carried the uvr1 mutation.

The dna9 uvr2 double mutant was constructed by the same procedure used to obtain the dna9 uvr1 combination, except transformation of streptomycin-resistant strain dna9 was carried out with DNA from an erythromycin-resistant strain DB116 (which carries the uvr2 mutation, linked to the erythromycin locus) and 320 erythromycin-resistant colonies were tested for UV sensitivity. Two UV-sensitive clones were obtained. Both were found to carry the dna9 and uvr2 mutations.

The previously observed linkage between the uvr1 gene and streptomycin resistance[76] and between the uvr2 gene and erythromycin resistance (J. E. LeClerc, unpublished data) was not observed here. In this work the uvr1 and uvr2 genes were being transferred into a uvr^+ strain. It was previously shown that the linkage between the uvr1 gene and the str, kan and nov markers was greatly reduced when uvr^+ cells were transformed to uvr1, compared to that obtained by transformation of the uvr1 strain with DNA from drug-resistant wild-type cells[76]. An analogous situation exists with respect to linkage between the rec1 gene and the streptomycin locus. When rec1 cells are transformed with DNA from streptomycin-resistant wild-type cells, a large fraction of the streptomycin-resistant transformants are rec^+. However, when the reverse transformation is performed, the apparent linkage between the rec1 and streptomycin loci is greatly reduced[112].

The dna9 mutation was moved into the UV-sensitive strain DB115 by transformation with DNA from streptomycin-resistant strain dna9, followed by replica plating at 37° and 40° C. Among approximately 5000 streptomycin-resistant transformant clones tested, one contained the dna9 mutation and a UV survival curve indicated that this strain still carried the UV sensitivity of the parent strain.

It was hoped that the dna9 mutation could be easily transferred to other H. influenzae strains by transformation with DNA from drug-resistant dna9, followed by selection for drug resistance controlled by a gene linked to the dna9 gene. It is evident from the transfer of the dna9 mutation into strain DB115 that the dna9 mutation is not closely linked to streptomycin resistance. Strain dna9 was made resistant to

kanamycin, dalacin, erythromycin, nalidixic acid and spectinomycin by transformation with DNA containing these drug markers. Using DNA from the multiply drug resistant dna9, the dna9 mutation was not co-transformed into wild-type cells along with any of these markers when 180 drug-resistant transformants were tested for temperature sensitivity.

Conditions of Growth

Liquid cultures were grown at 36°-37° C with aeration in 3.5% BHI (Difco Labs, Detroit, Michigan) supplemented with 10 µg per ml hemin and 3 µg per ml nicotinamide adenine dinucleotide (Sigma Chemical Co., St. Louis, Mo.). Solid medium for plating either under or on the surface contained 2.7% BHI, 0.75% Eugonbroth (BBL, Baltimore, Maryland) and 1.2% agar (Difco).

Irradiation of Cells with 313-nm Light

The method of 313-nm irradiation used in the selection of temperature sensitive DNA synthesis mutants was essentially as previously described[4]. Cells were centrifuged, washed with ice-cold M9 buffer (0.6% Na_2HPO_4, 0.3% KH_2PO_4, 0.05% NaCl and 0.1% NH_4Cl) and resuspended in the same buffer at 5 x 10^8 cells per ml (OD_{675} = 0.20). Cells (2.5 ml) were transferred to an ice-cooled quartz cuvette with a 1-cm light path and exposed to 313-nm light obtained by passing light from a Philips 1000-watt high pressure mercury arc lamp (Philips Electronic Instruments, Mount Vernon, New York) through a Hilger quartz prism monochromator (Hilger and Watts Inc., Morton Grove, Illinois) with a band width of 7 nm, then through a thin Mylar film (10% transmission at

308 nm, 1% at 306 nm) to eliminate scattered shorter wavelengths. The average fluence rates were determined using a bolometer (Yellow Springs Instrument Co., Yellow Springs, Ohio) calibrated against a standard lamp (National Bureau of Standards) and were corrected for absorption by the cell suspension. During the irradiation the cells were mixed with a Pasteur pipette at 2-minute intervals.

Treatment of Cells with NC

Streptomycin-resistant cells were grown to about 10^9 per ml in supplemented BHI, then centrifuged and resuspended at the same density in 0.05 M Tris-maleate buffer, pH 6.0. NC was then added and the cells were incubated at 36° C. The volume of cells, concentration of mutagen, and length of treatment for each experiment are given in the Results section. At the end of the treatment period the cells were diluted 1:2.5 into ice-cold BHI, washed with cold BHI and resuspended at 2.5 - 5 x 10^8 cells per ml in supplemented BHI.

Preparation of Lysates

At various times after treatment with mutagen, 10-ml aliquots of cells were removed to centrifuge tubes containing 10 ml ice-cold 1 M NaCl. The cells were centrifuged and resuspended at about 10^{10} cells per ml in 1 M NaCl containing 10^{-2} M ethylenediaminetetraacetate. Sodium dodecyl sulfate was then added at a final concentration of 0.125%, and after incubation at 37° C for 15 minutes the lysates were frozen.

Preparation of Competent Cells

Cells were made competent in M-IV medium by the method of Steinhart and Herriott[120].

Transformation with Lysates

After thawing, 0.5 ml lysate was added to 60 ml competent cells (streptomycin-sensitive and novobiocin-sensitive) and shaken for 30 minutes at 36° C before 180 ml supplemented BHI was added. After 2-3 hours of expression at 36°-37° C, 30 ml of transformed cells was added to 270 ml BHI growth medium containing streptomycin at a final concentration of 250 μg per ml. The streptomycin-resistant transformants were grown to stationary phase (OD_{675} = 0.8 - 1.0), then frozen in 3 ml aliquots for later measurement of the frequency of mutation to novobiocin resistance (which shows 30-50% cotransformation with streptomycin resistance). The large volumes of competent cells enabled detection of mutation frequencies to novobiocin resistance in the original mutagen-treated culture of 10^{-7} - 10^{-6}. Transformation frequencies ranged from 0.1% to 1%, averaging about 0.5%. In 60 ml competent cells there are about 10^{11} cells and, at 0.5% transformation, about 5 x 10^{8} streptomycin-resistant transformants. Thus, the 60 ml competent cells contained on the average approximately 20-200 cells destined to become resistant to novobiocin when the frequency of mutation to novobiocin was 10^{-7} - 10^{-6}.

Measurement of DNA Synthesis

DNA synthesis was measured as incorporation of (methyl-^{3}H)dThd (1 mCi per ml, 16 - 22 Ci per mmole, Schwartz/Mann, Orangeburg, New York). Samples of cells (0.5 ml) at 2.5 x 10^{8} per ml in BHI were added

to test tubes containing 20 µl (^{3}H)dThd and 20 µl inosine solution (10 mg per ml) and shaken at 36°-37° or 41° C. At various times 20-µl samples were removed and placed on 2.3 cm 3MM Whatman filter paper disks. The disks were washed three times (at least 5 minutes per wash) with ice-cold 5% TCA, washed three times with 95% ethanol, dried, and counted in a Packard Tri-Carb liquid scintillation spectrometer (Packard Instruments Co., Downers Grove, Illinois) using a toluene-BBOT scintillator.

Autoradiography

Incorporation of (^{3}H)dThd by individual cells was detected by the following procedure. Wild-type cells were treated with 5 µM NC for 3 minutes (18% survival), then washed and resuspended in growth medium at 2.5 x 10^8 cells per ml. To a tube containing 0.2 ml (^{3}H)dThd (1 mCi per ml, 2 Ci per mmole) and 0.1 ml inosine solution (10 mg per ml) was added 1.0 ml of cell suspension, and the tube was shaken at 37° C. After 60 minutes, 0.9 ml of cells was added to a tube containing 0.1 ml 40% formaldehyde, held on ice for 60 minutes, and then centrifuged, washed three times with 0.5% tryptone, and resuspended in 0.5% tryptone at 10^9 cells per ml. A drop of cells was placed onto a subbed microscope slide (previously dipped in a solution containing 0.5% gelatin and 0.01% chrome alum and then dried) and spread over the surface with the tip of a Pasteur pipette, and the slides were dried for 30 minutes at 50° on a slide warmer. The slides were rinsed with distilled water, washed three times with 5% TCA (5 minutes each) and three times with distilled water, and dried on the slide warmer. The slides were then dipped in Kodak NTB-2 emulsion (diluted 1:2), exposed at 4° C, developed in Kodak D-19 developer, and stained with Giemsa Azure A.

Alkaline Sucrose Gradient Centrifugation

To determine single-strand molecular weights of DNA of cells labeled before or after treatment with NC 0.10 ml 1N NaOH was layed onto each 5-ml linear 5-20% alkaline sucrose gradient[84] containing 0.1 M NaCl (pH 12), then approximately 10^8 cells in 0.1 ml M9 buffer were immediately layered onto each gradient. The gradients were made in 1/2 x 2 inch cellulose nitrate tubes (Beckman Instruments Inc., Fullerton, California). After 15 minutes at room temperature for lysis of cells on the gradients, centrifugation was carried out at 30,000 rpm at 13° C in a SW 50.1 rotor and Beckman model L ultracentrifuge (Beckman Instruments Inc.). DNA liberated from cells lysed on the gradients was centrifuged for 90 or 100 minutes. The distribution of radioactivity in the gradients was determined by puncturing the bottom of the tubes and collecting approximately 30 10-drop fractions on Whatman No. 17 paper strips[20]. After the strips were washed once with 5% TCA and twice with 95% ethanol and dried, they were cut into sections and the TCA-insoluble radioactivity was counted in a toluene-BBOT scintillator using a Packard Tri-Carb liquid scintillation spectrometer.

The data were put into a computer which calculated cross-over corrections and plotted sedimentation profiles, using a program written by M. L. Randolph, J. E. Donnellan and R. J. Brake.

N-Nitroso Compounds

MNNG was obtained from Aldrich Chemical Corporation, Milwaukee, Wisconsin. NC was kindly supplied by Dr. W. Lijinsky, Oak Ridge National Laboratory.

III. RESULTS

Mutation Fixation in the Absence of DNA Replication

A major aim of this investigation was to determine whether DNA replication is required for mutation fixation after treatment with N-nitroso compounds. Previous experiments[58] showing a reduction in mutation fixation under conditions of reduced DNA synthesis (in the presence of dBrUrd), suggested that it is.

The temperature-sensitive DNA synthesis mutant, streptomycin-resistant strain dna9, was treated with 1 μM NC for 6 minutes at 36° C, then washed twice and incubated in BHI growth medium at the permissive (36° C) and the nonpermissive (41° C) temperatures. Lysates made at various times were then used to transform wild-type cells to streptomysin resistance. The frequency of mutation to novobiocin resistance among the streptomycin-resistant transformants is a measure of the extent of mutation fixation. As seen in Figure 2, in the absence of DNA replication (41° C) some mutation fixation did occur, although much less than in the presence of DNA replication (36° C). This indicates that the bulk of the mutation fixation requires DNA replication, but that there is also a fixation process that is independent of DNA replication. A likely candidate for this nonreplicative type of mutation fixation is an error-prone repair system.

Figure 3 shows an experiment in which cells (96 ml) were treated with NC for 6 minutes at two different concentrations (1 μM and 10 μM), then mutation fixation was measured in the absence of DNA replication.

Figure 2. DNA synthesis and mutation fixation at the permissive temperature (36° C) and at the nonpermissive temperature (41° C) in streptomycin-resistant strain dna9 after treatment with NC.

Cells (64 ml) were treated with 1 μM NC for 6 minutes, then diluted 1:2.5 with ice-cold BHI, centrifuged, washed with cold BHI and resuspended at 2.5×10^8 cells per ml in supplemented BHI. The culture was divided in half and incubated at 36° and 41° C. Survival of the mutagen-treated cells was 53%. (A) Incorporation of (^{3}H)dThd. (o) Untreated control cells incubated at 36° C in BHI. (Δ) Cells treated with NC, then incubated in BHI at 36° C. (□) Cells treated with NC and incubated in BHI at 41° C. (B) Frequency of novobiocin-resistant mutants among the streptomycin-resistant transformants obtained after exposure of competent wild-type cells to lysates made at various times after treatment with mutagen. (Δ) Cells treated with NC, then incubated at 36° C in BHI for various times before lysates were made. (□) Mutagen-treated cells incubated in BHI at 41° C. (o) Untreated control cells incubated at 41° C in BHI.

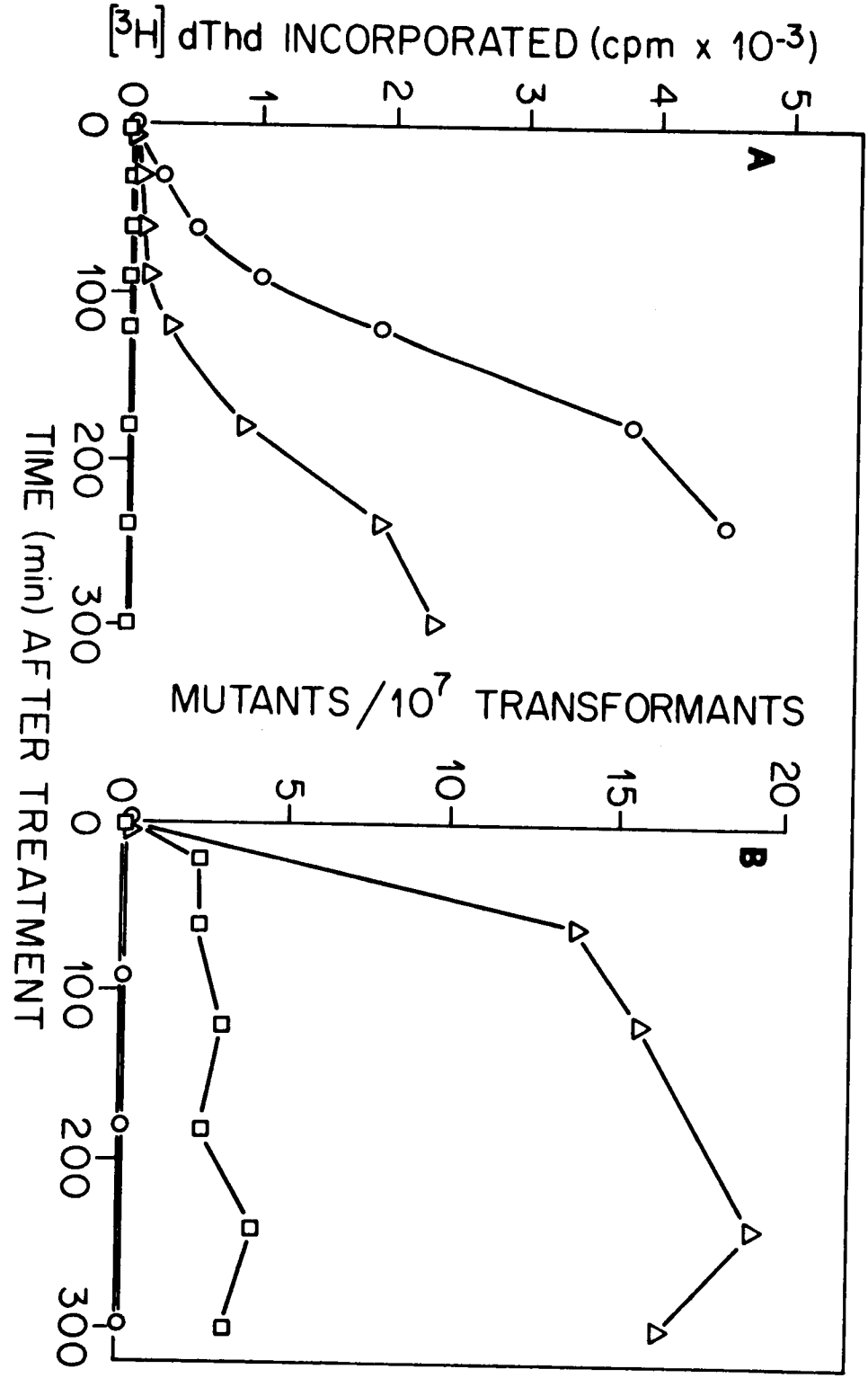

[3H] dThd INCORPORATED (cpm x 10-3)
0
1
2
3
4
5
A
0
100
200
300
TIME (min) AFTER TREATMENT
MUTANTS / 10^7 TRANSFORMANTS
0
5
10
15
20
B
0
100
200
300

Figure 2

Figure 3. Mutation fixation at 41° C after treatment of streptomycin-resistant strain dna9 with 1 μM (Δ) and 10 μM (▲) NC for 6 minutes.

Treated cells (100 ml at 5 x 10^8 per ml) were incubated at 41°C in BHI for various periods of time before lysates were made. The lysates were used to transform wild-type cells to streptomycin resistance, then the frequency of novobiocin-resistant mutants among the transformants was measured.

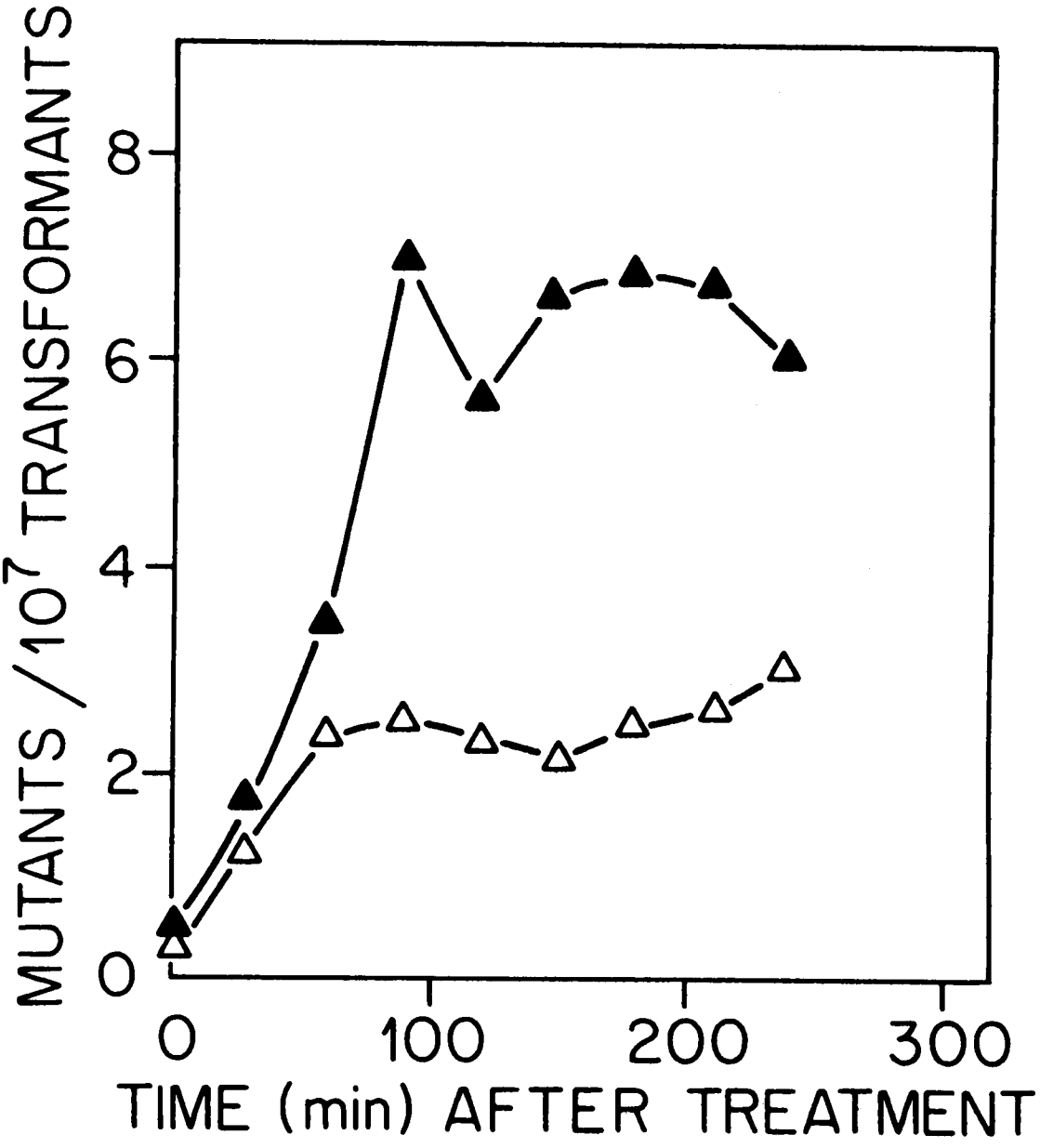

MUTANTS /10^7 TRANSFORMANTS
0
2
4
6
8
0
100
200
300
TIME (min) AFTER TREATMENT

Figure 3

The higher concentration of NC led to more mutation fixation at 41° C, indicating that the amount of mutation fixation at 41° C depends on the dose of mutagen, and therefore on the extent of mutagen-induced damage to the DNA.

Although the data of Figure 2 indicate that most of the mutation fixation requires DNA replication, they do not prove that replication is required throughout the entire period of mutation fixation. It is conceivable, for instance, that mutation fixation could involve error-prone repair of the DNA synthesized immediately after treatment with mutagen. To test the possibility that the requirement for DNA replication occurs only for a short time after treatment, the following experiment was done. The streptomycin-resistant strain dna9 was treated with 2 μM NC for 6 minutes, then washed and resuspended in BHI growth medium at 36° C. After 20 minutes of mutation fixation in the presence of DNA replication, half of the culture was shifted to 41° C to stop DNA replication, while the remainder was kept at 36° C. Lysates were made at various times for measurement of mutation fixation. If DNA replication were required for mutation fixation only immediately after treatment then the extent of mutation fixation should not be affected by incubation at 41° C after 20 minutes. However, as shown in Figure 4, the shift to 41° C at 20 minutes resulted in a substantial reduction in the amount of mutation fixation observed at later times. Thus, DNA replication is required for mutation fixation not only immediately after treatment, but also at later times.

Figure 4. Effect on subsequent mutation fixation of raising the temperature to 41° C after incubation of NC-treated streptomycin-resistant strain dna9 at 36° C for 20 minutes.

Cells (200 ml) were treated with 2 μM NC for 6 minutes, then washed and resuspended in BHI growth medium and incubated in BHI at 36° C for 20 minutes. Half of the culture was then incubated at 41° C (Δ) and the other half was kept at 36° C (□).

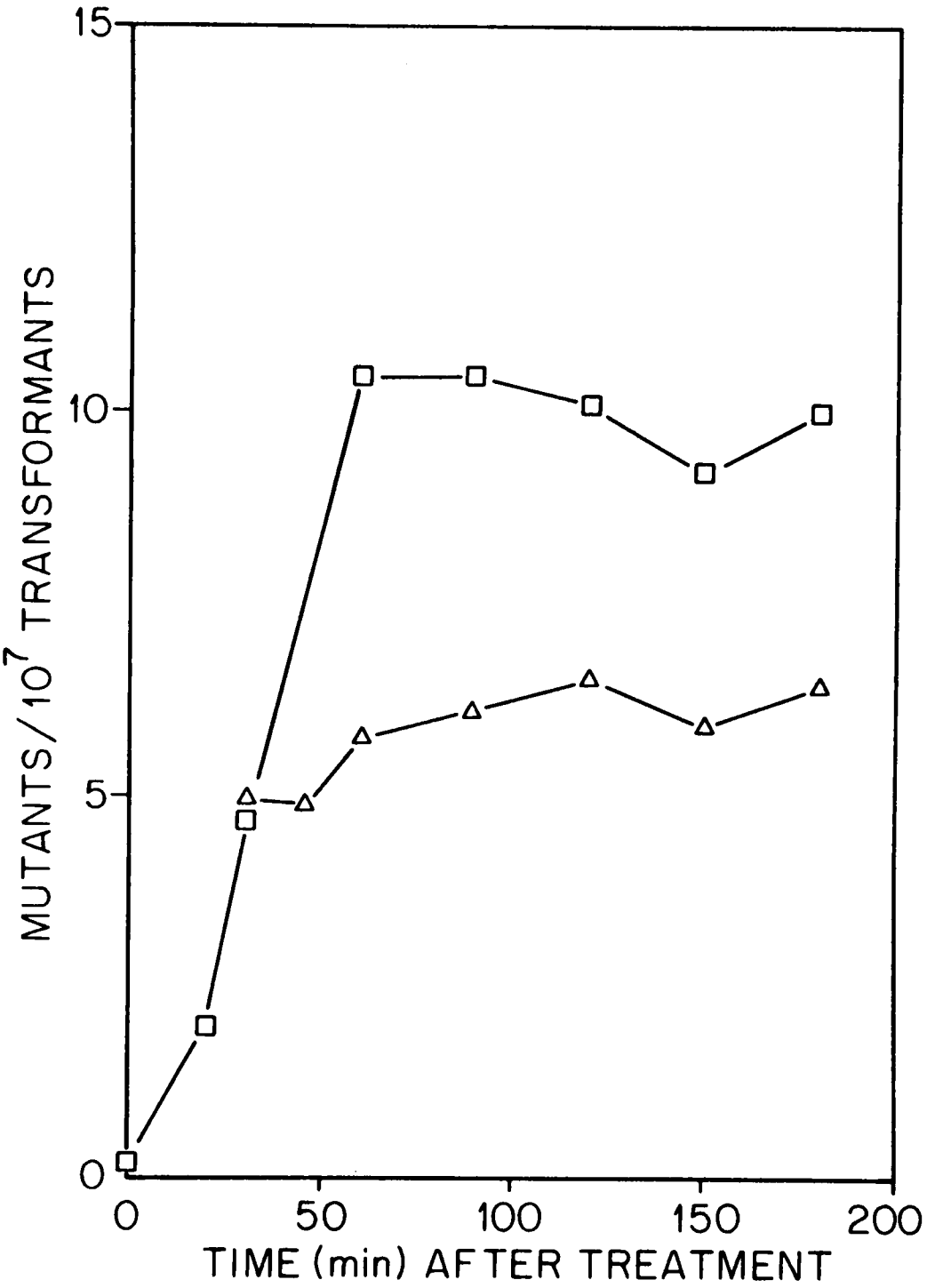

15
10
5
0
MUTANTS/10^7 TRANSFORMANTS
0
50
100
150
200
TIME (min) AFTER TREATMENT

Figure 4

Kinetics of DNA Replication and Mutation Fixation at 36° C After Treatment with NC

Previous experiments[58] demonstrating mutation fixation in H. influenzae cells after treatment with MNNG suggested that mutation fixation continues over most if not all of the first cell generation after treatment. Figure 5 shows a more detailed examination of the kinetics of mutation fixation at the permissive temperature after treatment of H. influenzae strain dna9 with NC (1 μM, 6 minutes). On the basis of optical density measurements (Figure 5A), the doubling time for the control cells was about 55 minutes, and that of the NC-treated cells was about 75 minutes. We cannot accurately estimate the rate of DNA synthesis in the treated cells from the optical density measurements, since the optical density of strain dna9 increases at a nearly normal rate for about one doubling time at the nonpermissive temperature. DNA synthesis was therefore measured by incorporation of (^{3}H)dThd (Figure 5B). It appears that the rate of DNA synthesis was sharply reduced in the treated cells.

The data do not indicate whether the incorporation of (^{3}H)dThd in the treated culture was due to slow DNA replication in all of the cells or to normal DNA replication in only a fraction of the cells. To distinguish between these two possibilities, incorporation of (^{3}H)dThd by individual cells was measured by autoradiography. Wild-type cells were treated with NC, grown for 60 minutes in the presence of (^{3}H)dThd, and prepared for autoradiography as described in the Materials and Methods section. The profiles of grain counts among treated and

Figure 5. Growth, DNA synthesis and mutation fixation during incubation of streptomycin-resistant H. influenzae strain dna9 in BHI at 36° C after treatment with NC.

Cells (96 ml) were treated with 1 μM NC for 6 minutes, then diluted 1:2.5 in cold BHI, centrifuged, washed with cold BHI and resuspended at 2.5×10^8 cells per ml in BHI growth medium and incubated at 36° C. Survival of mutagen-treated cells was 28%. (A) Optical density at 675 nm. (B) Incorporation of (^{3}H)dThd. (C) Frequency of novobiocin-resistant mutants among streptomycin-resistant transformants obtained after exposing competent wild-type cells to lysates made at various times after treatment with mutagen. (o) Untreated control cells. (Δ) Cells treated with NC. The dashed line indicates the frequency of novobiocin-resistant mutants obtained with a lysate made from untreated cells.

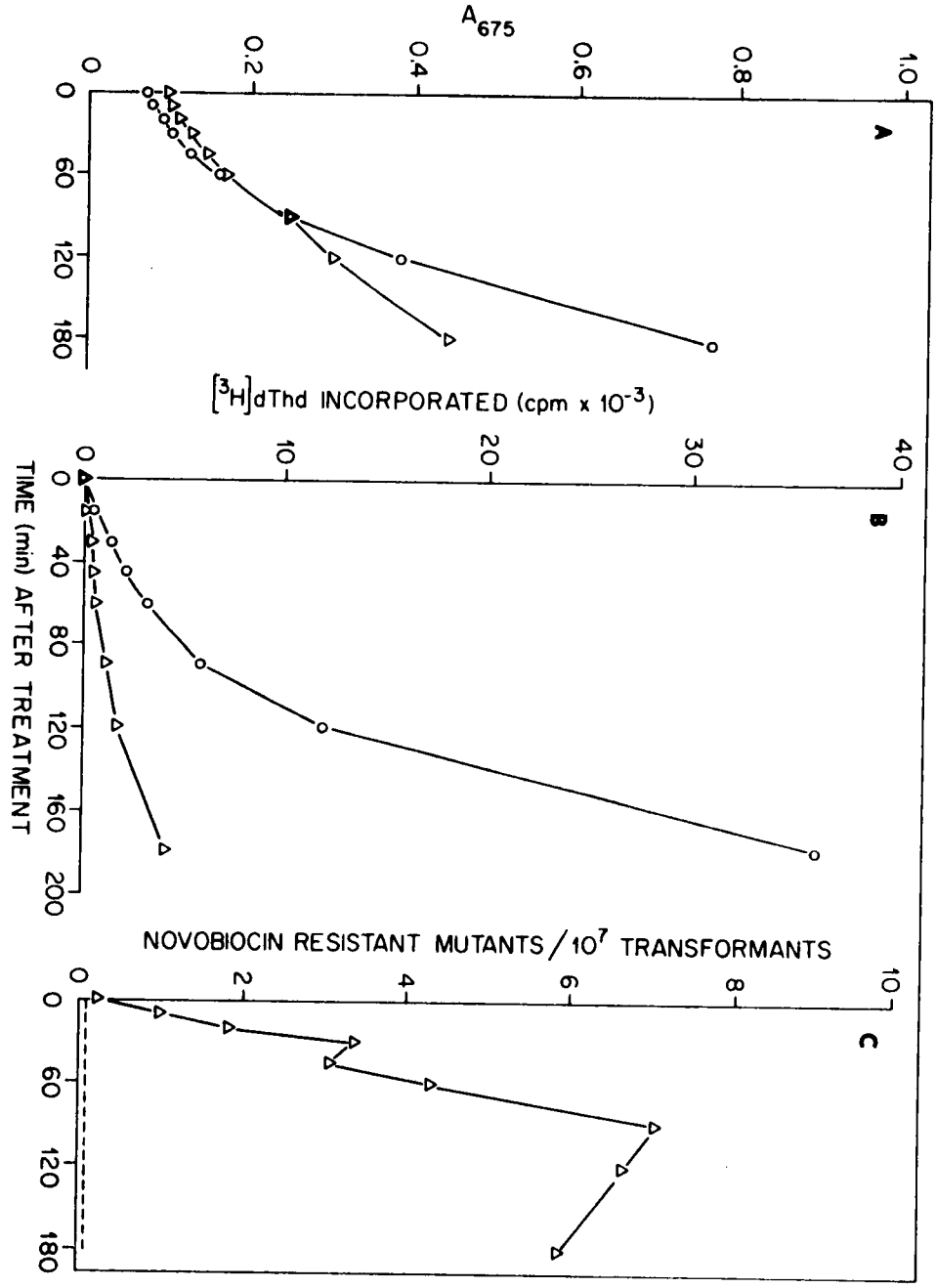

A675
0
0.2
0.4
0.6
0.8
1.0
A
0
60
120
180
[3H]dThd INCORPORATED (cpm x 10-3)
0
10
20
30
40
B
TIME (min) AFTER TREATMENT
0
40
80
120
160
200
NOVOBIOCIN RESISTANT MUTANTS / 10^7 TRANSFORMANTS
0
2
4
6
8
10
C
0
60
120
180

Figure 5

untreated cells (Figure 6) indicate that the vast majority of treated cells incorporated only about 25-35% as much as the control cells.

When corrections were made for survival and for differences in initial cell densities (see Figure 5A), it was calculated that it took an equivalent number of NC-treated cells about 170 minutes to incorporate the amount of (^{3}H)dThd that control cells incorporated during one cell doubling (55 minutes). Thus, in the majority of cells the first round of DNA replication was probably completed in about 170 minutes after treatment with NC. However, the profiles of grain counts in Figure 6 suggests that a fraction of treated cells (15-20% in the experiment of Figure 6) were synthesizing DNA at a nearly normal rate. It is conceivable that this fraction represents the surviving cells (18% in this experiment). Thus in the experiment shown in Figure 5 a fraction of the treated cells may have completed one round of DNA replication almost as soon as the untreated cells.

Figure 5C shows that mutation fixation, measured as an increase in the ability of the lysates made from the treated cells to transform a second culture to novobiocin resistance, increased rather linearly for about 60-90 minutes. It would be worthwhile to compare this with the length of time required to complete one round of DNA replication. However, this comparison is difficult to make if the treated cells actually consist of a major population synthesizing DNA at a greatly reduced rate and a minor population synthesizing DNA at a nearly normal rate. If 170 minutes is used as the length of time required to complete one round of DNA replication, then mutation fixation continued for 35-53% of the first round of DNA replication after treatment with mutagen. If about 25% of

Figure 6. Distribution of silver grains among individual cells of a population of wild-type H. influenzae treated with NC, grown in the presence of (^{3}H)dThd for 60 minutes, and prepared for autoradiography.

Autoradiographs were exposed for 14 days. (o) Untreated control cells. Silver grains in 794 cells counted. (Δ) Cells treated with 5 μM NC for 3 minutes (18% survival). Silver grains in 1697 cells counted.

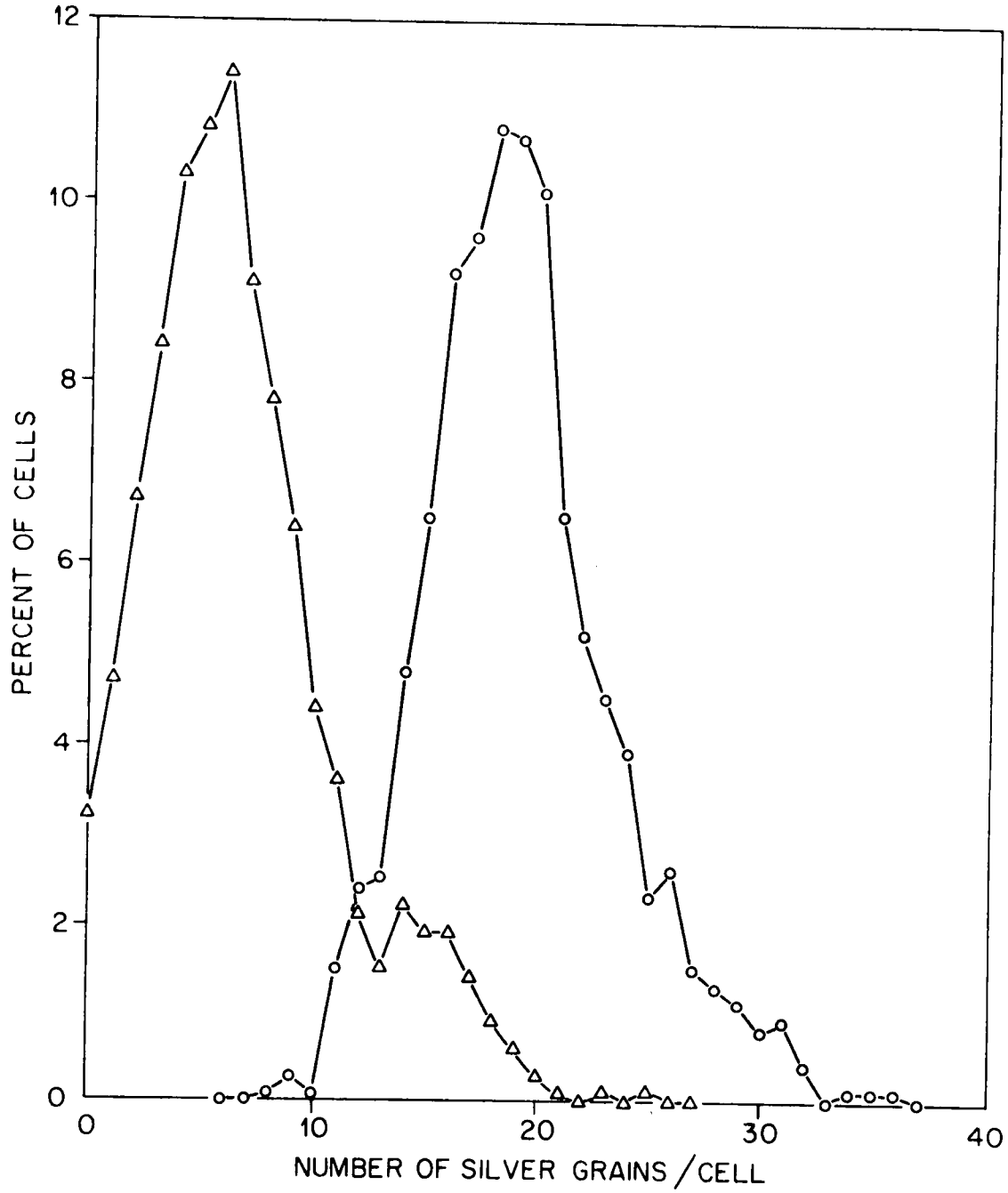

12
10
8
6
4
2
0
PERCENT OF CELLS
0
10
20
30
40
NUMBER OF SILVER GRAINS / CELL

Figure 6

the treated cells were synthesizing DNA at a nearly normal rate (completing the first round of replication in about 70 minutes) then since most of the mutation fixation requires DNA replication, this fraction of cells would account for a disproportionately large fraction of the mutation fixation. Nevertheless, it is likely that in the majority of cells mutation fixation continues for nearly half of the first round of DNA replication after treatment with NC.

Lack of Influence of the Defective Prophage on DNA Synthesis, Survival and Mutation Frequency in H. influenzae Treated with NC

H. influenzae strain *Rd* contains a defective prophage which is induced upon irradiation with small doses of UV light[119]. It was therefore important to determine whether a significant fraction of the incorporation of (^{3}H)dThd observed after treatment with NC was caused by induction of the defective prophage. Wild-type strain *Rd* and strain BC200, which lacks an inducible defective prophage[3, 6], were treated with 1 μM NC for 6 minutes, then survival was measured and the cells were washed and resuspended in BHI growth medium. Incorporation of (^{3}H)dThd was measured in a small sample of the cells and the remaining cells were grown to stationary phase for measurement of the frequency of novobiocin-resistant mutants. It is clear from the data of Figure 7 that the presence of an inducible defective prophage had no effect on incorporation of (^{3}H)dThd after treatment with NC. There was also no effect on survival (62% for *Rd*, 63% for BC200), nor on mutation frequency (1.86×10^{-6} for *Rd*, 1.71×10^{-6} for BC200).

Figure 7. Incorporation of (^{3}H)dThd in wild-type strain Rd and in strain BC200 during incubation of cells in BHI at 36°C after treatment with 1 μM NC for 6 minutes.

Open symbols represent untreated cells and closed symbols represent treated cells. (o,●) Strain Rd, 62% survival. (Δ,▲) Strain BC200, 63% survival.

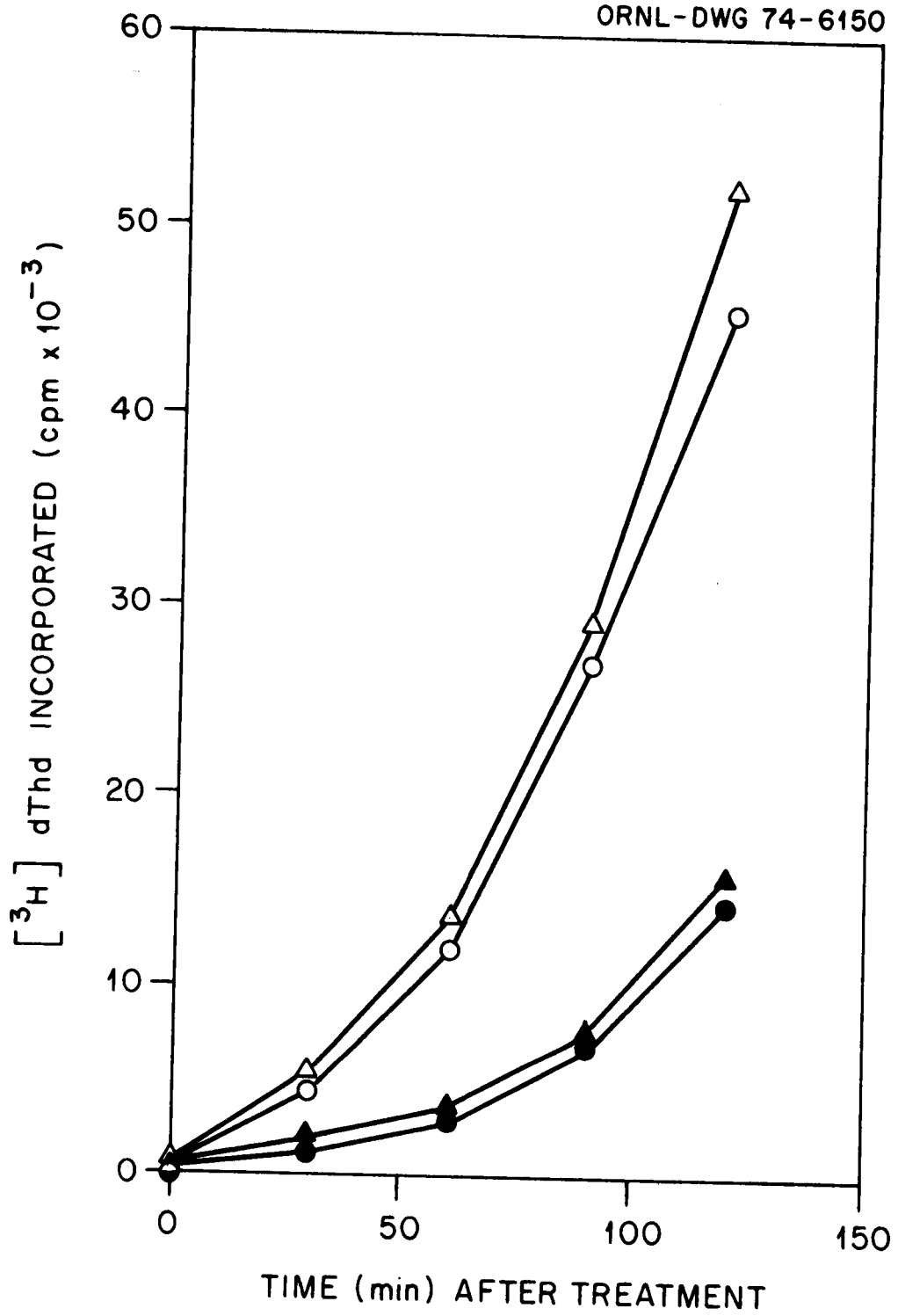

ORNL-DWG 74-6150
60
50
40
30
20
10
0
[3H] dThd INCORPORATED (cpm x 10-3)
0
50
100
150
TIME (min) AFTER TREATMENT

Figure 7

Evidence for Repair of Premutational Lesions in H. influenzae Cells Treated with N-Nitroso Compounds

Repair of mutagen-induced damage to the DNA could have two effects:

1. An error-prome repair mechanism could lead to alteration of the nucleotide sequence.
2. Repair of premutational lesions before the replication fork reaches them could act to decrease the final yield of mutation.

We have attempted to detect repair of premutational lesions in the following way. The temperature-sensitive DNA synthesis mutant, dna9, was treated with NC (1 μM, 6 minutes) at 36° C, then washed and incubated in BHI at 41°C for various periods of time to allow repair in the absence of replication-dependent mutation fixation, then grown at 36° C for at least three generations before the frequency of mutation to novobiocin resistance was measured. As seen in Figure 8A, the final mutation yield decreased as a function of the time that the cells were held at 41° C before transfer to 36° C. In preliminary experiments this decrease was also seen after treatment with MNNG. These data suggest that some repair mechanism may operate to remove premutational lesions in the absence of DNA replication. It is not absolutely certain that this decrease in mutation yield was caused by repair, since the possibility has not been ruled out that the decrease may have been caused by some nonenzymatic process, such as loss of alkylated purines from the DNA.

Repeated experiments indicated that after the final mutation frequency has fallen to 20-30% of the original level (after 40-60 minutes

Figure 8. Final mutation frequency and rate of DNA synthesis after incubation of NC-treated dna9 cells at 41° C for various times before the cells were grown at 36° C.

Cells (96 ml) were treated with 1 μM NC for 6 minutes at 36° C, then washed and resuspended at 5 x 10^8 cells per ml in supplemented BHI at 41° C. After various times aliquots were diluted 1:2 in supplemented BHI and grown for at least three generations before 3-ml aliquots were frozen. (A) Decrease in final frequency of mutation to novobiocin resistance with increasing period of incubation of cells at 41° C between mutagen treatment and cell growth. The size of the samples that were shifted to 36° C after various times at 41° C was adjusted to counteract the loss of viability at 41° C (10 ml at 0 minutes, 20 ml at 20 minutes, 40 ml at 40 minutes, and 80 ml at 60 minutes). (▲) Experiment 1; survival of NC-treated cells was 51%. (Δ) Experiment 2; survival of NC-treated cells was 53%. (B) Incorporation of (^{3}H)dThd after incubation of NC-treated cells (experiment 1 of A) in BHI at 41° C for 0 (●), 20 (□), 40 (Δ), and 60 (o) minutes before being diluted 1:2, shifted to 36° C, and incubated with (^{3}H)dThd.

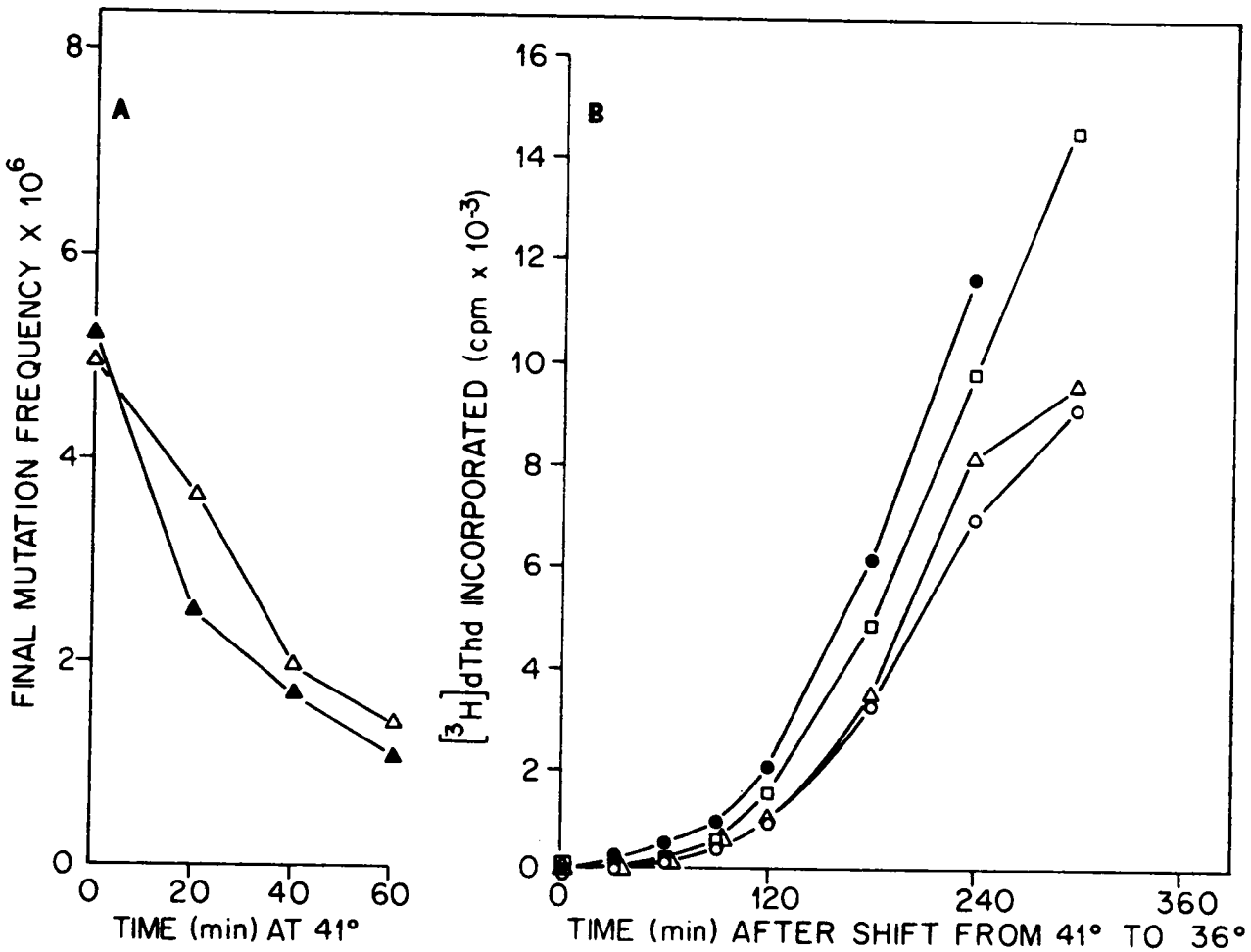

A
FINAL MUTATION FREQUENCY X 10^6
8
6
4
2
0
0
20
40
60
TIME (min) AT 41°
B
[3H]dThd INCORPORATED (cpm x 10^-3)
16
14
12
10
8
6
4
2
0
0
120
240
360
TIME (min) AFTER SHIFT FROM 41° TO 36°

Figure 8

at 41° C), there is no further decrease. The remaining level of mutation may reflect mutation fixation occurring in the absence of DNA replication.

Interpretation of this experiment depends on the assumption that DNA synthesis resumed upon shifting the temperature from 41° to 36° C. Figure 8B shows incorporation of (^{3}H)dThd by NC-treated cells held at 41° C for 0, 20, 40 and 60 minutes before the temperature was shifted to 36° C and (^{3}H)dThd was added (same experiment as Figure 8A). DNA synthesis did in fact resume in the treated cells after the temperature was decreased to 36° C. The separation between the curves in Figure 8B may be due to loss of survival in dna9 at 41° C. This loss is somewhat variable from one experiment to the next, but does not depend on mutagen treatment.

Effect of the rec1 Mutation on Survival and Mutation Frequency in H. influenzae Treated with NC

It was previously reported that strain DB117 of H. influenzae, which carries the rec1 mutation, is sensitive to killing by monofunctional alkylating agents[59] but that the frequency of mutation to novobiocin resistance is similar in strain DB117 and wild-type strain Rd after treatment with MNNG. Figure 9 shows survival of strains dna9 and dna9 rec1 after treatment with increasing concentrations of NC for 6 minutes. The presence of the rec1 mutation clearly causes an increased sensitivity to the lethal effect of the compound.

Mutation frequency was also measured in these two strains after treatment with increasing concentrations of NC for 6 minutes. The results, shown in Figure 10, indicate that the frequency of mutation to

Figure 9. Survival of H. influenzae strains dna9 (Δ) and dna9 rec1 (o) after exposure to various concentrations of NC.

Cells were treated with NC for 6 minutes at 36° C, then diluted with 1:100 in ice-cold BHI and plated immediately.

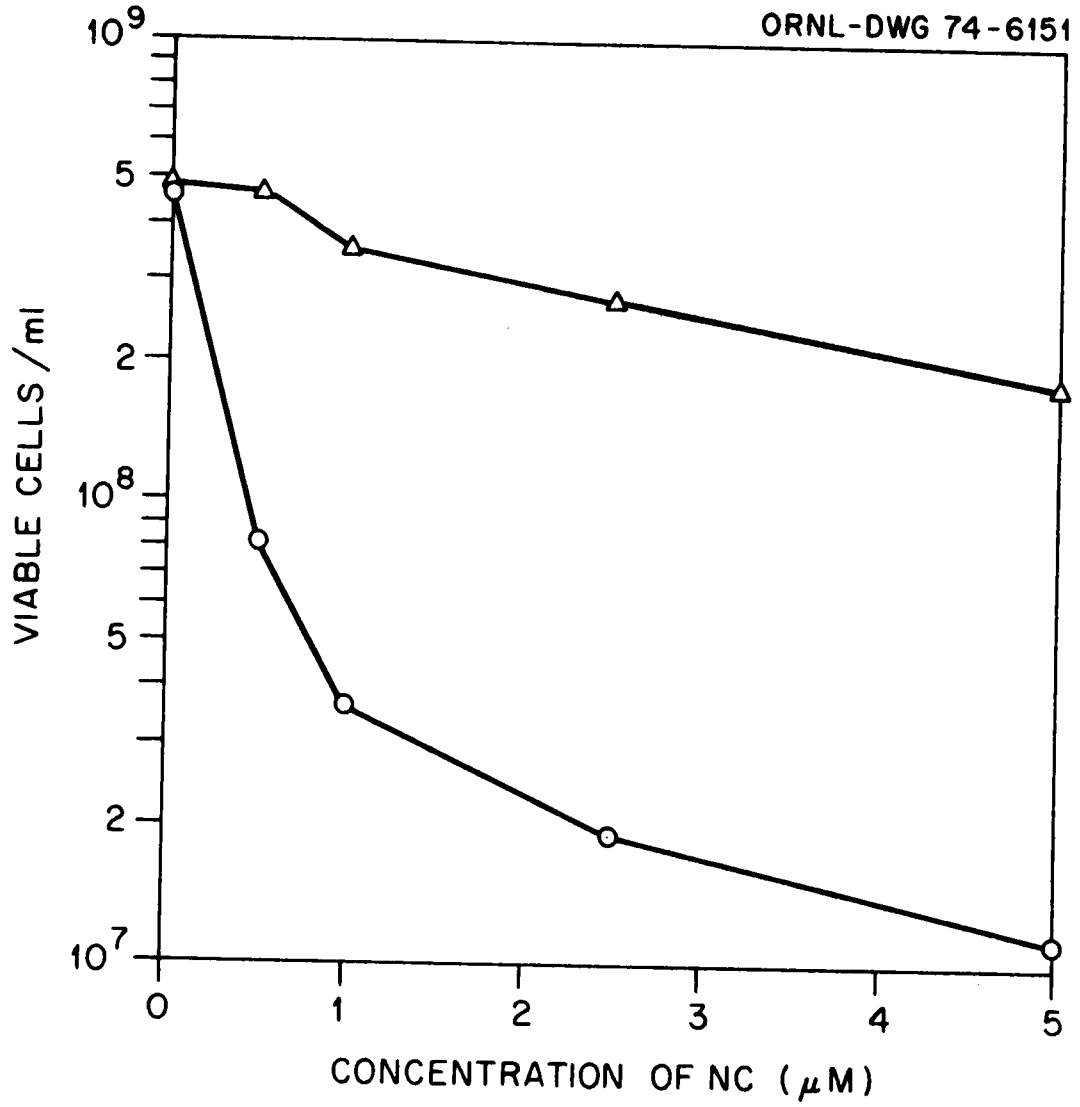

ORNL-DWG 74-6151
VIABLE CELLS/ml
10^9
5
2
10^8
5
2
10^7
0
1
2
3
4
5
CONCENTRATION OF NC (μM)

Figure 9

Figure 10. Frequency of mutation to novobiocin resistance in H. influenzae strains dna9 (Δ) and dna9 rec1 (o) after exposure to various concentrations of NC.

Cells (20 ml dna9 or 20-100 ml dna9 rec1, depending on the concentration of mutagen) were treated at approximately 10^9 per ml in Tris-maleate buffer, pH 6, for 6 minutes at 36° C, then diluted 1:2.5 in cold BHI, centrifuged, washed with cold BHI, resuspended at four times the original volume in supplemented BHI, grown to early stationary phase (OD_{675} 0.8 - 1.0), and frozen in 3-ml aliquots. After appropriate dilution thawed cells were plated for measurement of mutation frequency as described in the Materials and Methods section.

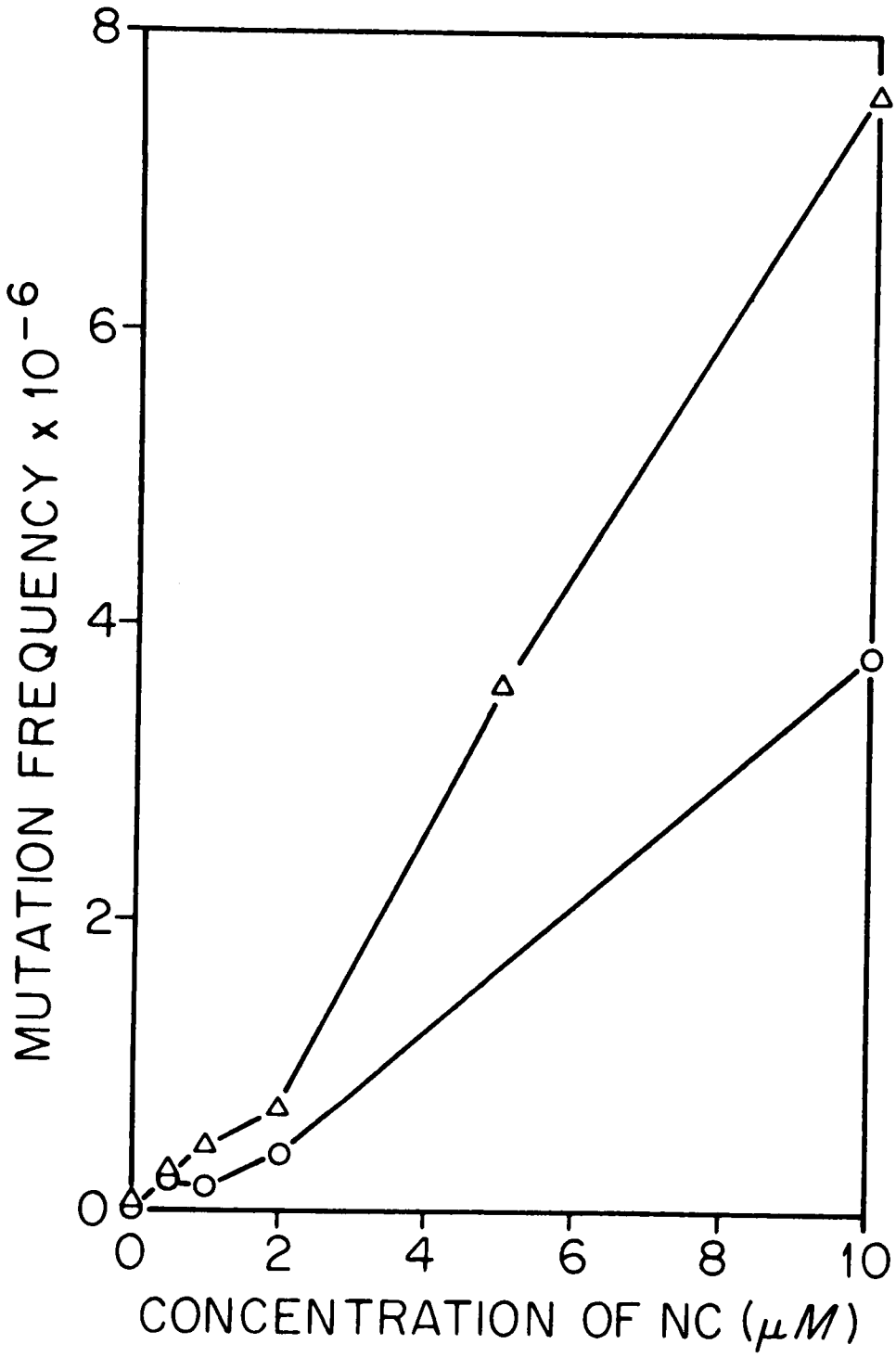

MUTATION FREQUENCY x 10⁻⁶
8
6
4
2
0
0
2
4
6
8
10
CONCENTRATION OF NC (μM)

Figure 10

novobiocin resistance is higher in strain dna9 than in strain dna9 rec1. Repeated experiments suggest that besides causing reduced NC-induced mutagenesis, the rec1 mutation also confers a two to five fold increase in spontaneous mutability

It was shown in Figure 2 (p. 15) that mutation fixation consists of a component that requires DNA replication and a component that is independent of DNA replication. An experiment was therefore done to determine whether the rec1 mutant lacks the nonreplicative type of mutation fixation. Figure 11 shows mutation fixation at 41° C in strains dna9 and dna9 rec1 after treatment with 10 μM NC for 6 minutes. The presence of the rec1 mutation clearly does not eliminate the nonreplicative component of mutation fixation. The final mutation frequencies obtained when the cells were grown at 36° C were also measured in the experiments shown in Figure 11. For strain dna9 the final mutation frequency was 4.75×10^{-6}, whereas for strain dna9 rec1 it was 1.26×10^{-6}. By comparing these figures with the level of mutation fixation observed in the absence of DNA replication (Figure 11) an estimate can be made of the fraction of mutation that can be accounted for by nonreplicative mutation fixation. To make this estimate the levels shown in Figure 11 should be doubled, since only about half of the novobiocin-resistant mutants are being detected. (There is about 50% linkage between the str and nov markers in a lysate). When this is done it appears that all of the mutation in the rec1 mutant can be accounted for by the nonreplicative type of mutation fixation. However, in the dna9 strain lacking the rec1 mutation, only 25-30% of the mutation can be accounted for by the nonreplicative component of

Figure 11. Mutation fixation at 41° C after treatment of H. influenzae strains dna9 (Δ) and dna9 rec1 (o) with 10 μM NC for 6 minutes.

Treated cells (100 ml at 5 x 10^8 per ml) were incubated at 41° C in supplemented BHI for various times before being lysed. The lysates were used to transform wild-type cells to streptomycin resistance, then the frequency of novobiocin-resistant mutants among the transformants was measured. The data for strain dna9, shown here for comparison, were obtained in the experiment of Figure 3 (p. 17).

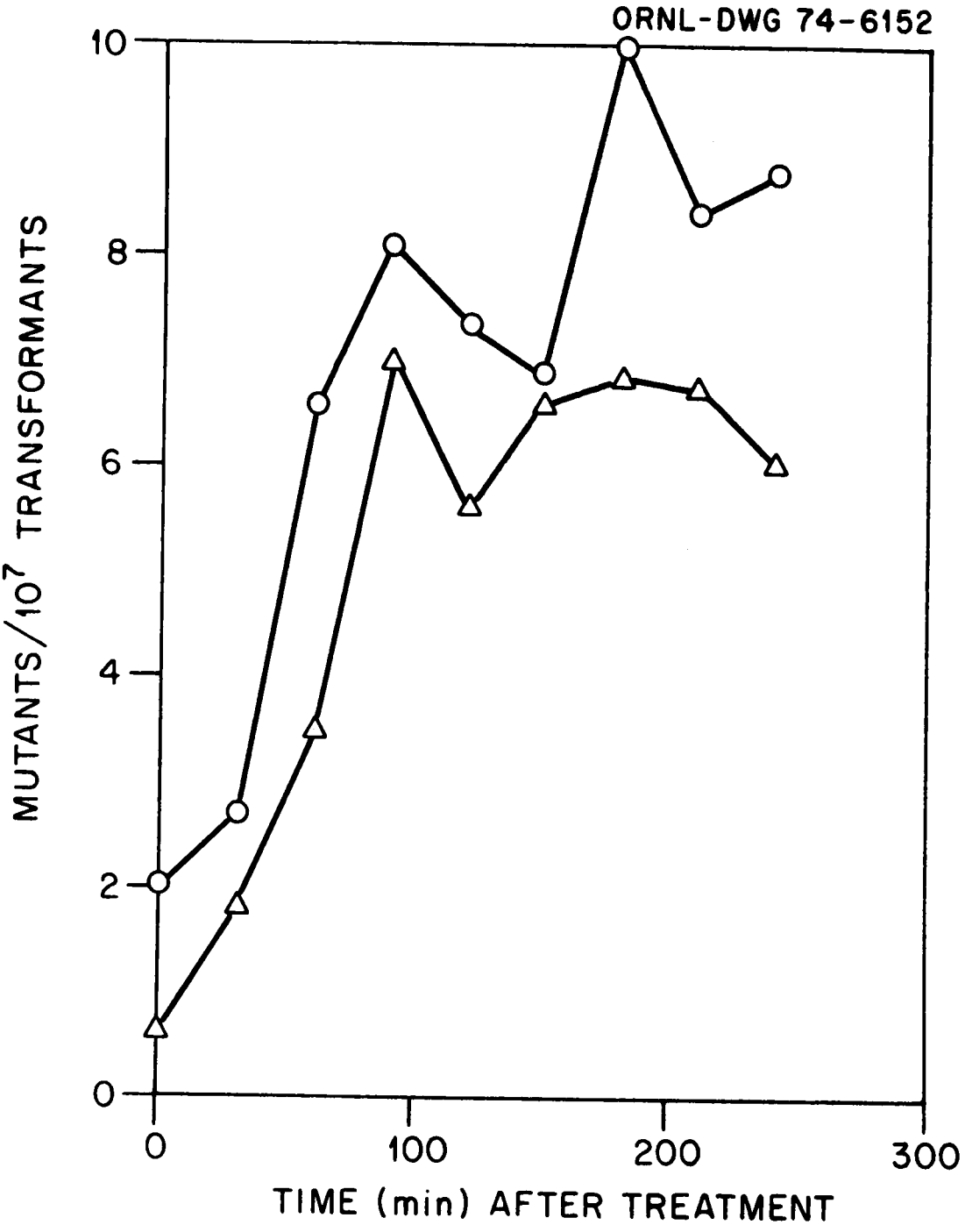

ORNL-DWG 74-6152
MUTANTS/10^7 TRANSFORMANTS
10
8
6
4
2
0
0
100
200
300
TIME (min) AFTER TREATMENT

Figure 11

mutation fixation. This is consistent with the data of Figure 2 (p. 15), where mutation fixation at 41° C was only about 20% as great as at 36° C.

Another indication that there is a deficiency in the replication-dependent mode of mutation fixation in the recl strain is the fact that little or no repair of premutational lesions was observed in strain dna9 recl. When this strain was treated with 2 μM NC for 6 minutes, then incubated at 41° C for 60 minutes before growth at 36° C, the mutation frequency was 2.7×10^{-7}, compared with 3.3×10^{-7} in cells grown entirely at 36° C. Little if any repair of premutational lesions should be observable in a strain in which mutation occurs only by nonreplicative fixation, since this type of mutation fixation proceeds in the absence of replication.

It appears, therefore, that the reduced NC-induced mutagenesis in the recl strain (Figure 10) can be explained by a deficiency in the replication-dependent component of mutation fixation in the recl mutant. If this is true, then the amount of mutation fixation in the recl mutant would be expected to be similar in the presence and absence of DNA replication. Figure 12 shows the results of two separate experiments in which mutation fixation was measured in NC-treated strain dna9 recl at 36° and 41° C. Although the levels of mutation fixation at 36° and 41° C are similar at late times, the initial period of mutation fixation at 36° in the recl mutant looks very much like that in the wild-type strain. Whereas in the wild-type strain the mutations fixed at 36° are stable (see Figures 2 and 4, pp. 15 and 20), most of the mutations fixed in the recl strain disappear at later times. The

Figure 12. Mutation fixation at the permissive (36° C) and nonpermissive (41° C) temperatures in streptomycin-resistant strain dna9 recl after treatment with NC.

Cells (100 ml) were treated with 2 μM (A) or 10 μM (B) NC for 6 minutes, then diluted 1:4 in cold BHI, centrifuged, washed with cold BHI and resuspended in 200 ml supplemented BHI at either 36° C (o) or 41° C (Δ). At various times lysates were made and used to transform wild-type cells to streptomycin resistance, then the frequency of novobiocin-resistant mutants among the transformants was measured.

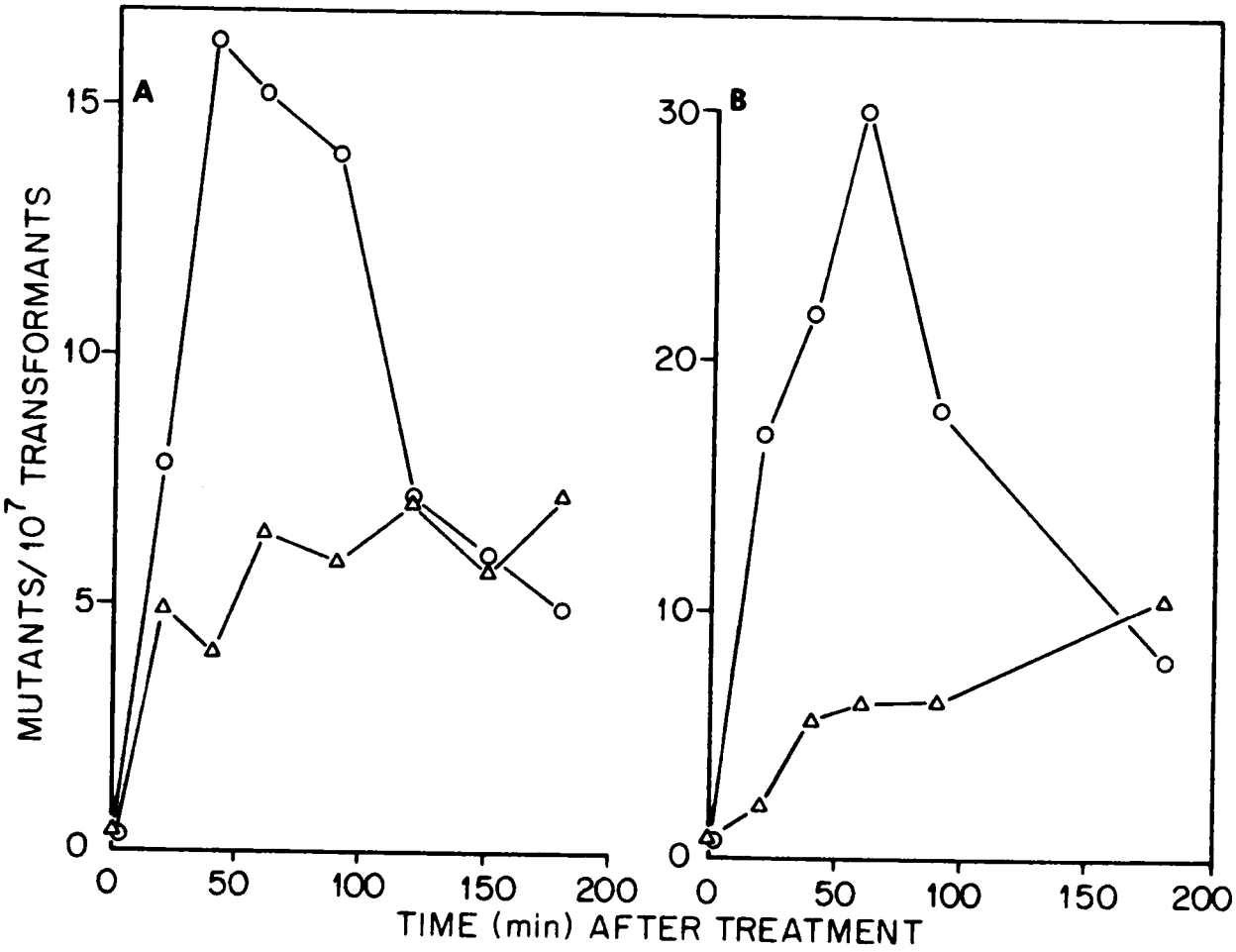

A
B
MUTANTS/ 10^7 TRANSFORMANTS
TIME (min) AFTER TREATMENT
0
5
10
15
20
30
50
100
150
200

Figure 12

disappearance of newly fixed mutations in the recl strain begins at about the time that mutation fixation reaches a maximum in the wild-type strain.

To compare the amount of mutation fixation in the wild-type and recl strains and to determine whether the disappearance of the newly fixed mutations in the recl strain requires further DNA replication the following experiment was done. Streptomycin-resistant strains dna9 and dna9 recl were treated with 1 μM NC for 6 minutes, then mutation fixation was allowed to proceed in the presence of DNA replication for 40 minutes. The bulk of the treated cells was then shifted to 41° C to stop DNA replication, while part was kept at 36° C. Lysates were made at various times up to 180 minutes and used to transform a second culture to streptomycin resistance. The frequency of novobiocin-resistant mutants among the streptomycin-resistant transformants is shown in Figure 13. At early times (up to 40 minutes after treatment) mutation fixation occurred to the same extent in strains dna9 and dna9 recl. The shift to the nonpermissive temperature did not prevent the disappearance of the newly fixed mutations in the recl strain. Although the kinetics of disappearance of newly fixed mutations in the recl strain at 36° C were not followed in this experiment, the decrease did occur at 36° C (as in Figure 12), since the level of mutation remaining at 180 minutes after treatment was similar whether the culture was shifted to 41° C at 40 minutes or kept at 36° C for the full 180 minutes (see Figure 13 legend).

Figure 13. Mutation fixation in NC-treated H. influenzae strains dna9 (Δ) and dna9 rec1 (o) during incubation in growth medium for 40 minutes at 36° C, then for 140 minutes at 41° C.

Streptomycin-resistant cells (100 ml) were treated with 1 μM NC for 6 minutes, then washed and resuspended in 200 ml BHI growth medium at 36° C. Lysates made at various times after treatment were used to transform wild-type cells to streptomycin resistance, then the frequency of novobiocin-resistant mutants among the transformants was measured. Lysates made from treated cells grown at 36° C for the entire 180 minute period gave mutation frequencies of 9.7×10^{-7} (for dna9) and 8.6×10^{-8} (for dna9 rec1).

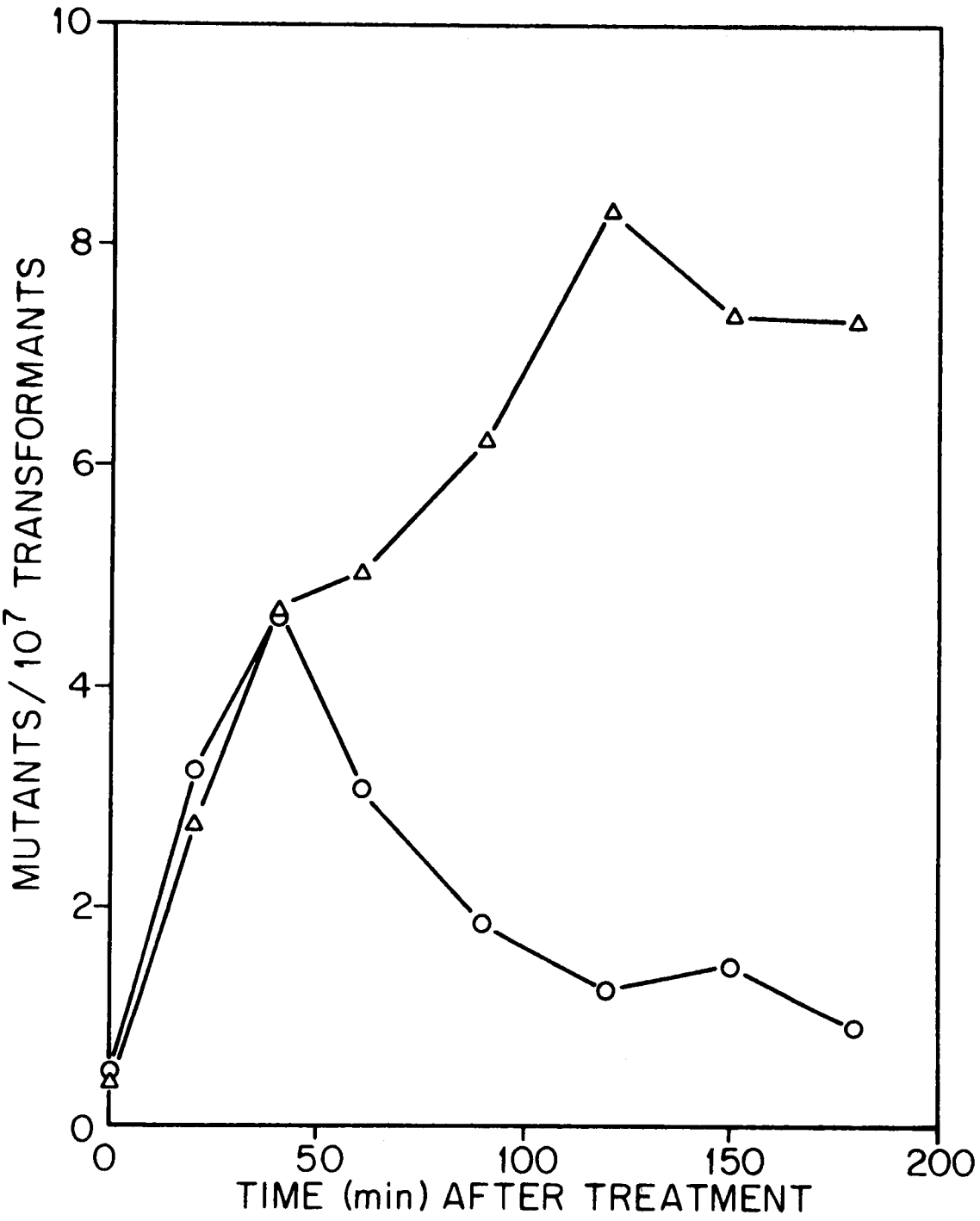

10
8
6
4
2
0
MUTANTS/10^7 TRANSFORMANTS
0
50
100
150
200
TIME (min) AFTER TREATMENT

Figure 13

State of DNA Synthesized Before and After Treatment of Strains dna9 and dna9 rec1 with NC

Experiments were carried out to determine whether the disappearance of newly fixed mutations in dna9 rec1 could be correlated with breakdown or lack of repair of DNA synthesized either before or after treatment with NC. It was previously reported that there are single strand breaks or alkali-labile bonds formed in prelabeled DNA of wild-type and rec1 strains of H. influenzae after treatment with MNNG[54]. Some breakdown of the prelabeled DNA was seen in both strains after treatment with MNNG, and evidence was presented that indicated that at least some of the MNNG-induced single strand breaks or alkali-labile bonds were repaired, in both wild-type and rec1 strains[54]. Strains dna9 and dna9 rec1 were both labeled with (^{14}C)dThd or (^{3}H)dThd for three generations. The (^{3}H)dThd-labeled cells were treated with 1 μM NC for 6 minutes, then washed and resuspended in BHI growth medium containing 500 μg per ml of unlabeled dThd. The untreated (^{14}C)dThd-labeled cells were resuspended in the same medium and mixed with an equal number of treated, ^{3}H-labeled cells. The cells were grown at 36° C and samples were taken at various times for measurement of TCA-insoluble radioactivity remaining and for measurement of single-strand molecular weights on alkaline sucrose gradients. As seen in Figure 14, there is degradation of prelabeled DNA in both strains after treatment with NC although the degradation is greater in the strain carrying the rec1 mutation.

Sedimentation analysis of the DNA liberated on top of alkaline sucrose gradients is shown in Figure 15. Immediately after treatment

Figure 14. Percent of acid-insoluble radioactivity (^{3}H) remaining after incubation of prelabeled, NC-treated strains dna9 (Δ) and dna9 rec1 (o) in BHI at 36° C.

At O. $D._{675}$ 0.05, 1.0 ml cells in BHI was added to a tube containing 0.05 ml inosine solution (10 mg per ml) and 0.10 ml (^{3}H)dThd (100 μCi/ml, 17 Ci/mmole), and grown at 36° C to $O.D._{675}$ 0.40. The cells were then centrifuged, resuspended in Tris-maleate buffer, pH 6, and treated with 1 μM NC for 6 minutes, then washed with cold BHI and resuspended in 4 ml supplemented BHI containing 500 μg per ml unlabeled dThd. Control cells (1 ml) were added to a tube containing 0.05 ml inosine solution (10 mg per ml) and 0.10 ml (^{14}C)dThd (50 μCi/ml, 62 mCi/mmole) and labeled from $O.D._{675}$ 0.05 to 0.40, then incubated for 6 minutes in Tris-maleate buffer without mutagen, centrifuged, washed and resuspended in 4 ml supplemented BHI containing 500 μg per ml unlabeled dThd. The ^{14}C- and ^{3}H-labeled cells were mixed and incubated at 36° C for various periods of time before 50-μl samples were taken for measurement of acid-insoluble radioactivity and 1-ml samples were taken for analysis of the DNA by alkaline sucrose gradient centrifugation.

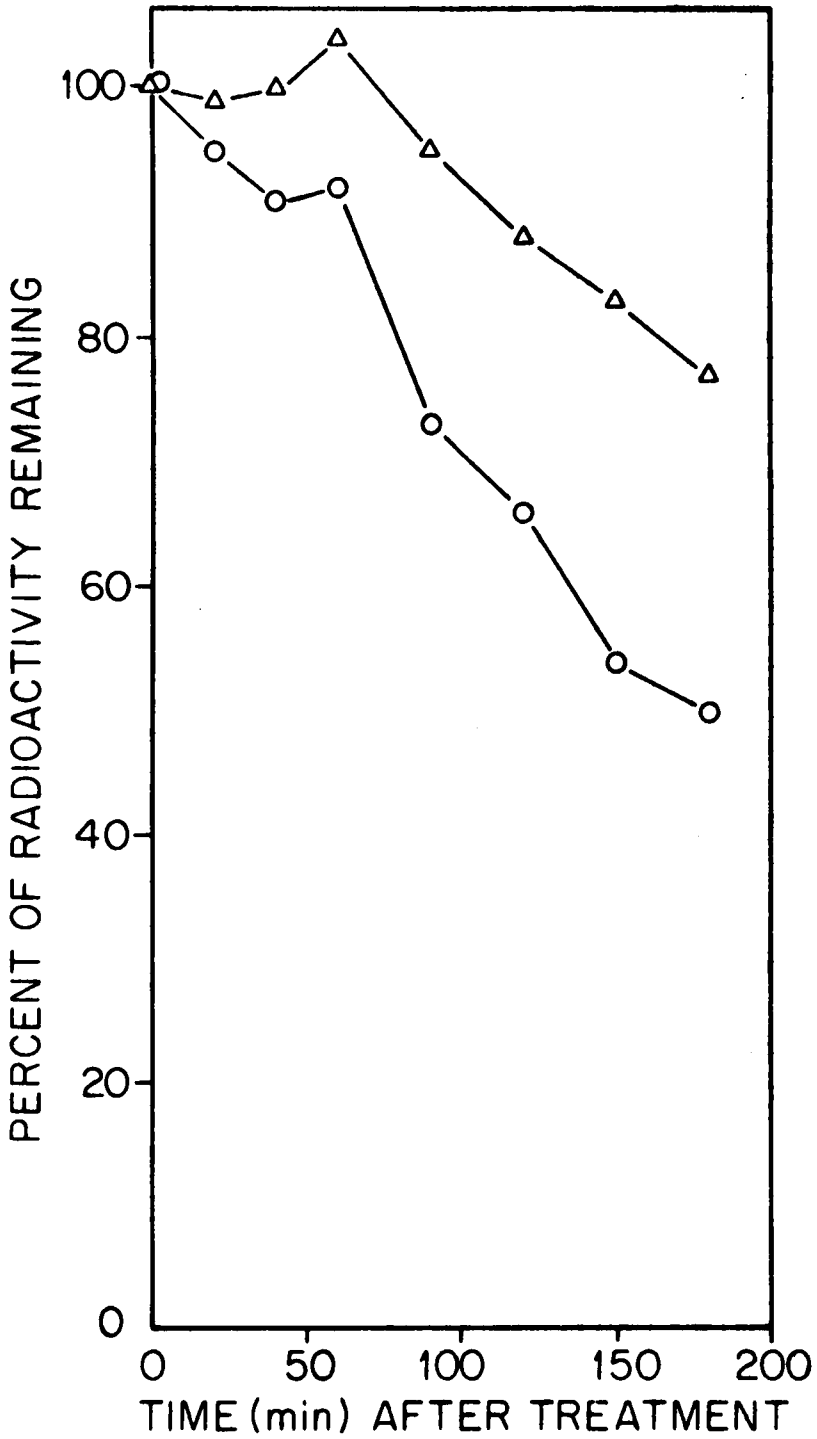

PERCENT OF RADIOACTIVITY REMAINING
100
80
60
40
20
0
0
50
100
150
200
TIME (min) AFTER TREATMENT

Figure 14

Figure 15. Alkaline sucrose gradient sedimentation profiles of DNA from prelabeled, NC-treated strains dna9 and dna9 rec1 after incubation in BHI for various periods of time at 36° C.

Experimental procedure explained in Figure 14. Gradients were centrifuged at 30,000 rpm for 90 minutes. (Δ) ^{14}C-labeled DNA of untreated cells. (o) ^{3}H-labeled DNA of NC-treated cells.

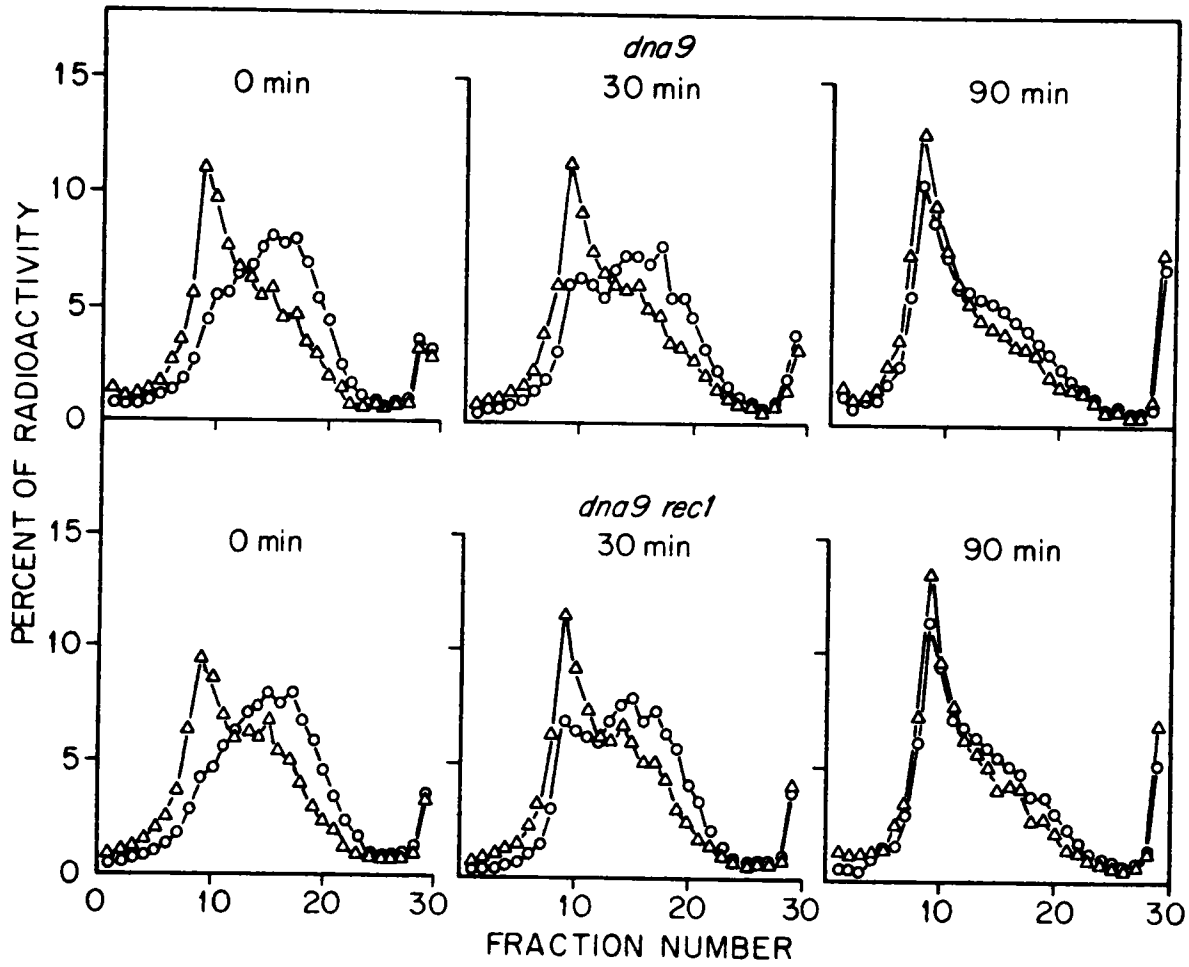

dna9
0 min
30 min
90 min
dna9 rec1
0 min
30 min
90 min
PERCENT OF RADIOACTIVITY
FRACTION NUMBER
0
5
10
15
0
10
20
30

Figure 15

with NC single-strand breaks or alkali-labile bonds were present, as evidenced by the decreased single-strand molecular weight of DNA from treated cells, compared to that from untreated cells. In both dna9 and dna9 rec1 there was some return of the ^{3}H-labeled DNA to the position of the control DNA after 30 minutes, and after 90 minutes there was almost complete return of the ^{3}H-labeled DNA to the position of the control DNA. It is therefore clear that repair of NC-induced single strand breaks or alkali-labile bonds occurs in H. influenzae, and is not prevented by the rec1 mutation.

In a second type of experiment the fate of DNA synthesized immediately after treatment of dna9 and dna9 rec1 cells with NC was examined. Control cells were labeled with (^{14}C)dThd for three generations and NC-treated cells were labeled with (^{3}H)dThd for 15 minutes after treatment. After being resuspended in BHI growth medium containing 500 µg per ml of cold dThd, the ^{14}C-labeled and ^{3}H-labeled cells were mixed and grown at 36° C. Samples were taken at various times for measurement of TCA-insoluble radioactivity remaining and for determination of single-strand molecular weights on alkaline sucrose gradients. As seen in Figure 16, degradation of DNA (^{3}H-labeled) synthesized immediately after treatment with mutagen occurred to a much greater extent in the strain carrying the rec1 mutation.

The sedimentation analysis on alkaline sucrose gradients of DNA synthesized after treatment with 1 µM NC for 6 minutes is shown in Figure 17. The DNA synthesized immediately after treatment with NC sediments more slowly than the prelabeled DNA, in both dna9 and dna9

Figure 16. Percent of acid-insoluble radioactivity (^{3}H) remaining in NC-treated strains dna9 (Δ) and dna9 rec1 (o) labeled for 15 minutes after treatment and then incubated in nonradioactive BHI for various periods of time at 36° C.

Cells were treated with 1 μM (A) or 2 μM (B) NC for 6 minutes, then washed and resuspended in BHI at $O.D._{675}$ 0.20. To a tube containing 0.20 ml (^{3}H)dThd (1 mCi/ml, 17 Ci/mmole) and 0.10 ml inosine solution (10 mg per ml) was added 2.0 ml treated cells. After incubation for 15 minutes at 36° C the cells were centrifuged and resuspended in 4 ml BHI containing 500 μg per ml unlabeled dThd. Control cells (1.0 ml) at $O.D._{675}$ 0.05 were added to a tube containing 0.10 ml (^{14}C)dThd (50 μCi/ml, 62 mCi/mmole) and 0.05 ml inosine solution (10 mg per ml) and labeled for three generations, then centrifuged and incubated at 36° C in Tris-maleate buffer, pH 6, for 6 minutes, then centrifuged and resuspended in 4 ml BHI containing 500 μg per ml unlabeled dThd. ^{14}C-labeled untreated cells were mixed with ^{3}H-labeled treated cells and incubated at 36° C for various periods of time before 50-μl samples were taken for measurement of acid-insoluble radioactivity and 1-ml samples were taken for analysis of the DNA by alkaline sucrose gradient centrifugation.

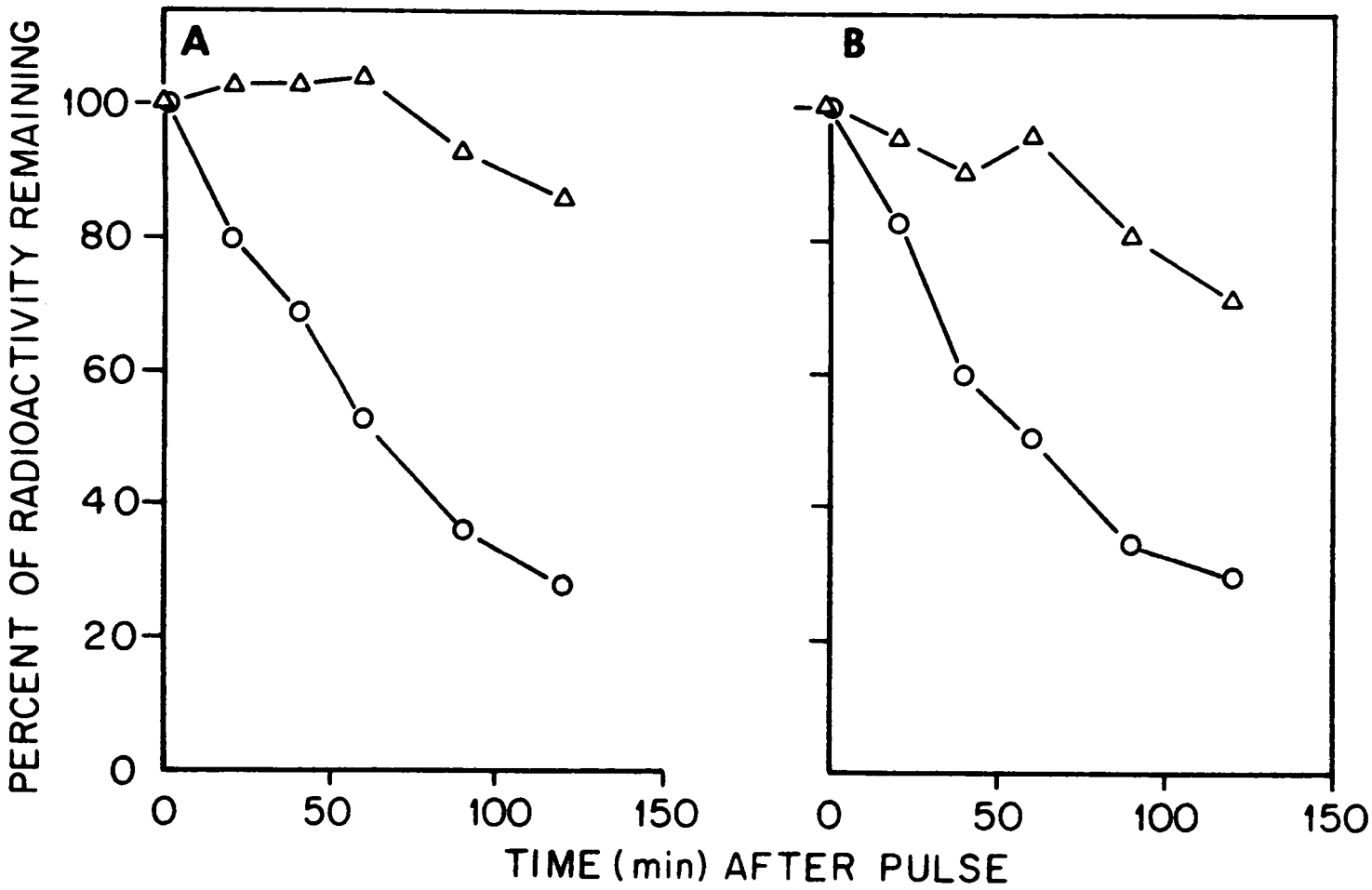

A
B
PERCENT OF RADIOACTIVITY REMAINING
100
80
60
40
20
0
0
50
100
150
0
50
100
150
TIME (min) AFTER PULSE

Figure 16

Figure 17. Alkaline sucrose gradient sedimentation profiles of DNA from strains dna9 and dna9 rec1 treated with 1 μM NC for 6 minutes, then labeled for 15 minutes before incubation in nonradioactive BHI at 36° C for various periods of time.

The experiment was that of Figure 16A. Gradients were centrifuged at 30,000 rpm for 90 minutes. (Δ) ^{14}C-labeled DNA of untreated cells. (o) ^{3}H-labeled DNA of NC-treated cells.

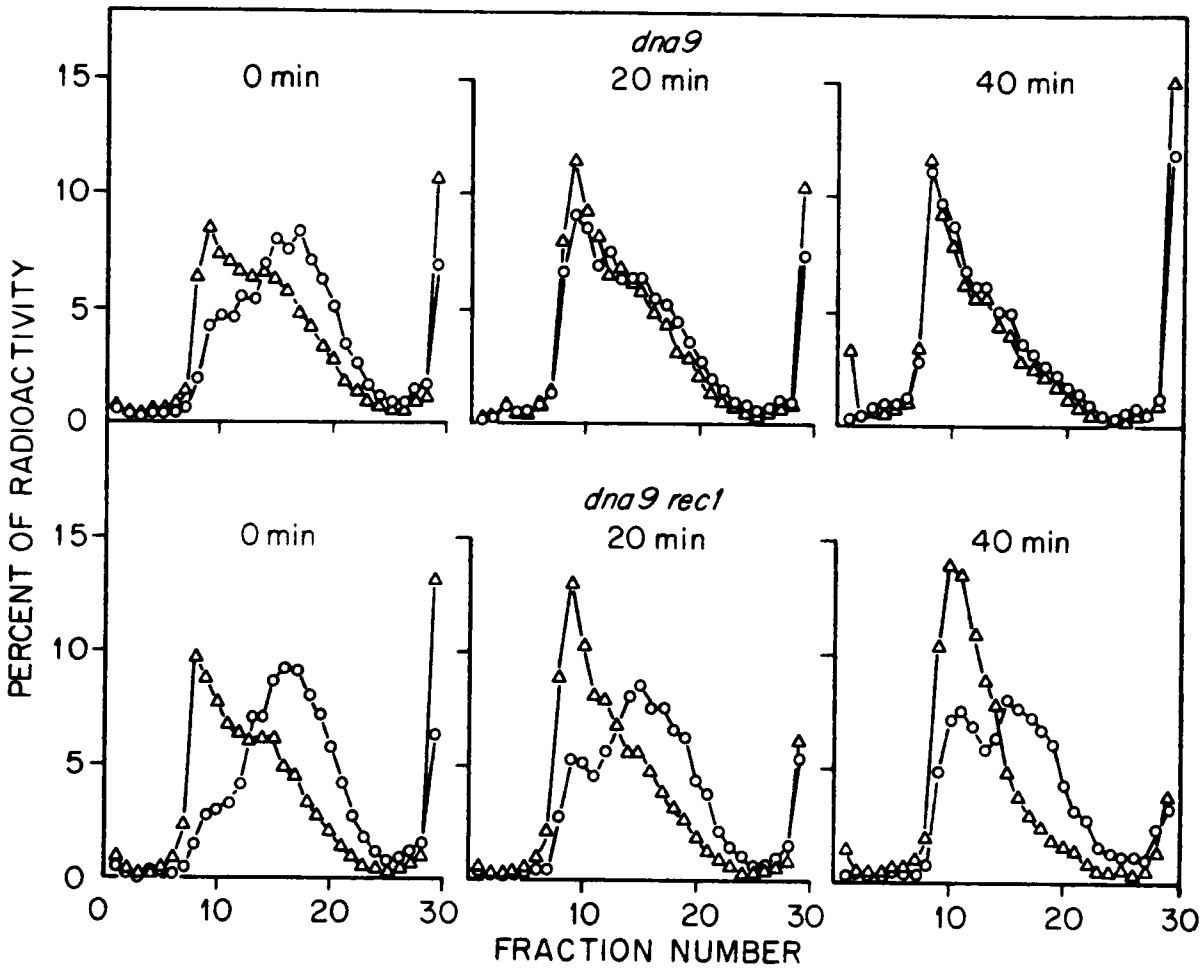

dna9
0 min
20 min
40 min
dna9 rec1
0 min
20 min
40 min
PERCENT OF RADIOACTIVITY
FRACTION NUMBER
0
5
10
15
0
10
20
30

Figure 17

recl. In strain dna9 there is a rapid shift in molecular weight of the ^{3}H-labeled DNA to the position of the ^{14}C-prelabeled DNA. This phenomenon is essentially complete in strain dna9 by 20 minutes. In strain dna9 recl, on the other hand, the smaller molecular weight ^{3}H-labeled DNA is shifted to the position of the prelabeled DNA much more slowly. The apparent slow shift of the newly synthesized DNA in NC-treated recl cells to the position of the control DNA is not due to preferential degradation of DNA of lower molecular weight, because there was a substantial increase in the absolute number of tritium counts at the position of the control DNA in the case of recl cells. Furthermore, at 40 minutes after the tritium pulse in recl cells, almost half of the tritium counts appear at the higher molecular weight position, while only about 30% of the newly synthesized DNA has been degraded to acid-soluble products. The fact that the molecular weight of the pulse labeled DNA from recl cells did not decrease appreciably during the subsequent incubation suggests that some DNA molecules were rapidly and completely degraded, while others underwent little or no degradation during the period examined.

The increase with time of the molecular weight of newly synthesized DNA in NC-treated cells is similar to the phenomenon seen in Bacillus subtilis cells treated with methyl methanesulfonate (MMS)[102] and resembles the "postreplication repair" phenomenon seen in UV-irradiated bacteria. The recl mutant was previously shown to be deficient in postreplication repair after UV irradiation[75].

Figure 18 shows the sedimentation analysis on alkaline sucrose gradients of DNA synthesized after treatment with 2 μM NC for 6 minutes.

Figure 18. Alkaline sucrose gradient sedimentation profiles of DNA from strains dna9 and dna9 rec1 treated with 2 μm NC for 6 minutes, then labeled for 15 minutes before incubation in nonradioactive BHI at 36° C for various periods of time.

The experiment was that of Figure 16B. Gradients were centrifuged at 30,000 rpm for 100 minutes. (Δ) ^{14}C-labeled DNA of untreated cells. (o) ^{3}H-labeled DNA of NC-treated cells.

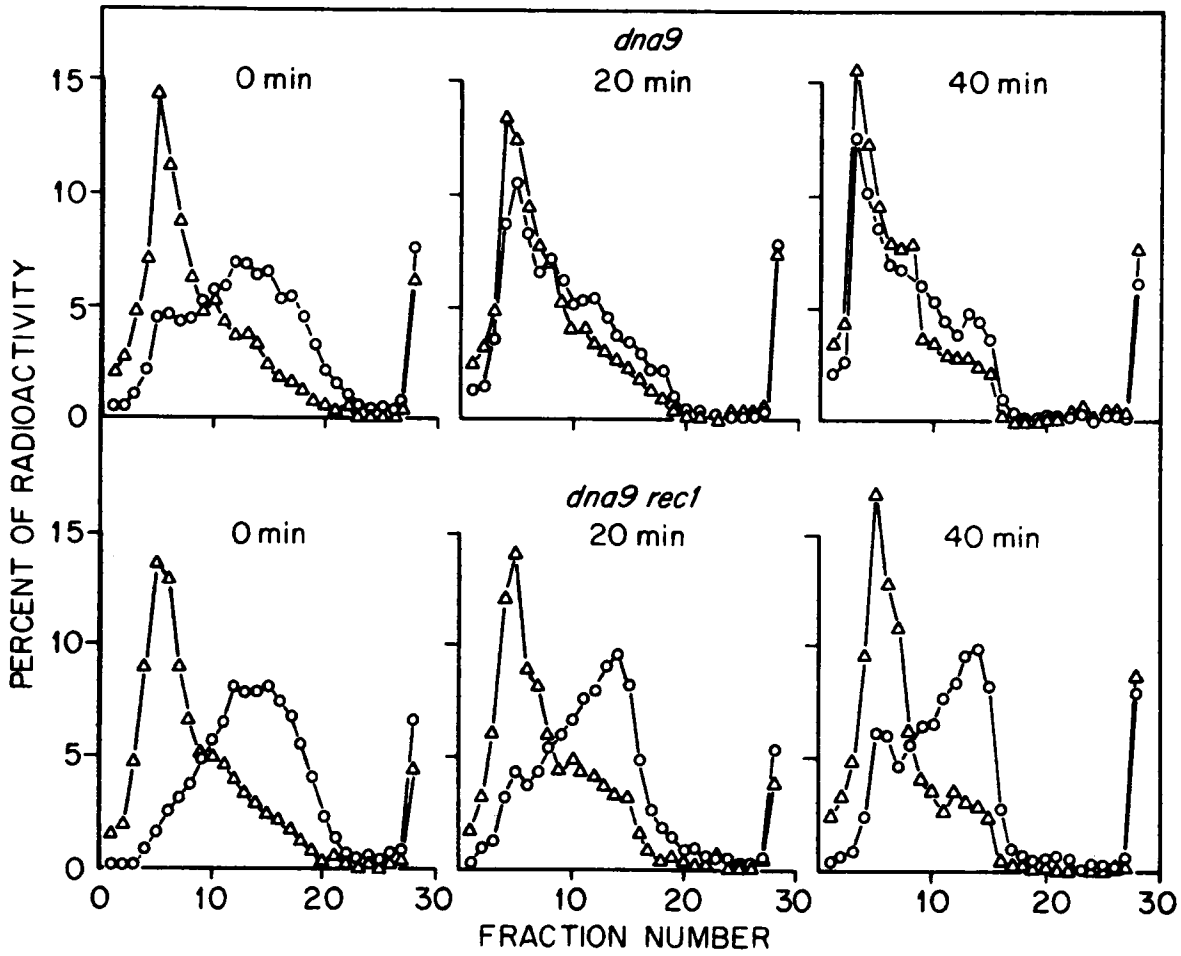

dna9
0 min
20 min
40 min
dna9 rec1
0 min
20 min
40 min
PERCENT OF RADIOACTIVITY
FRACTION NUMBER
0
5
10
15
0
10
20
30

Figure 18

In wild-type cells the strand joining phenomenon is complete within 20 minutes, but in recl cells it occurs even more slowly than in cells treated at the lower concentration of NC.

Nonreplicative Mutation Fixation and Repair of Premutational Lesions in H. influenzae Strains Deficient in Various Steps in the Excision-Repair of UV-induced Pyrimidine Dimers

To determine what type of cellular mechanisms are involved in the nonreplicative type of mutation fixation and repair of premutational lesions it would be helpful to obtain mutants defective in these processes. One approach to this objective would be to check for these phenomena in the 30-40 repair- and recombination-defective H. influenzae mutants that have been previously isolated[5, 13] (and K. L. Beattie, J. K. Setlow, and J. E. LeClerc, unpublished data). Accordingly, the dna9 mutation has been combined with several of these other mutations and the resulting strains have been tested for mutation fixation at 41° C and repair of premutational lesions.

The uvrl mutation of H. influenzae (carried by strain DB112) is believed to involve a defect in the UV-endonuclease that makes the initial incision adjacent to a pyrimidine dimer in the DNA of UV-irradiated cells[111]. Figure 19A shows that the uvrl mutation prevents neither nonreplicative mutation fixation, nor repair of premutational lesions. Thus, the UV-endonuclease of H. influenzae is apparently not involved in either of these processes.

Strains of H. influenzae carrying the uvr2 mutation (e.g., DB116) are unable to excise UV-induced pyrimidine dimers because of a

Figure 19. Mutation fixation and disappearance of premutational lesions resulting from treatment of three different UV-sensitive strains of H. influenzae with 2 μM NC for 6 minutes, then incubation in the absence of DNA replication for various periods of time.

Cells at 10^9 per ml in 300 ml Tris-maleate buffer, pH 6, were treated with 2 μM NC for 6 minutes, then washed and resuspended at 5 x 10^8 per ml in supplemented BHI at 41° C. After various times 20-ml samples were removed for preparation of lysates and aliquots (20 ml at 0 minutes, 50 ml at 20 minutes, 100 ml at 40 minutes, 200 ml at 60 minutes) were diluted 1:2 in supplemented BHI and grown for at least three generations before 3-ml aliquots were frozen for later measurement of mutation frequency. (o) Final frequency of mutation to novobiocin resistance in cells held at 41° C for various periods of time between treatment with NC and growth at 36° C. (Δ) Frequency of novobiocin-resistant mutants among streptomycin-resistant transformants obtained after exposure of competent wild-type cells to lysates made at various times after treatment. (A) Strain dna9 uvr1. (B) Strain dna9 uvr2. (C) Strain dna9 DB115.

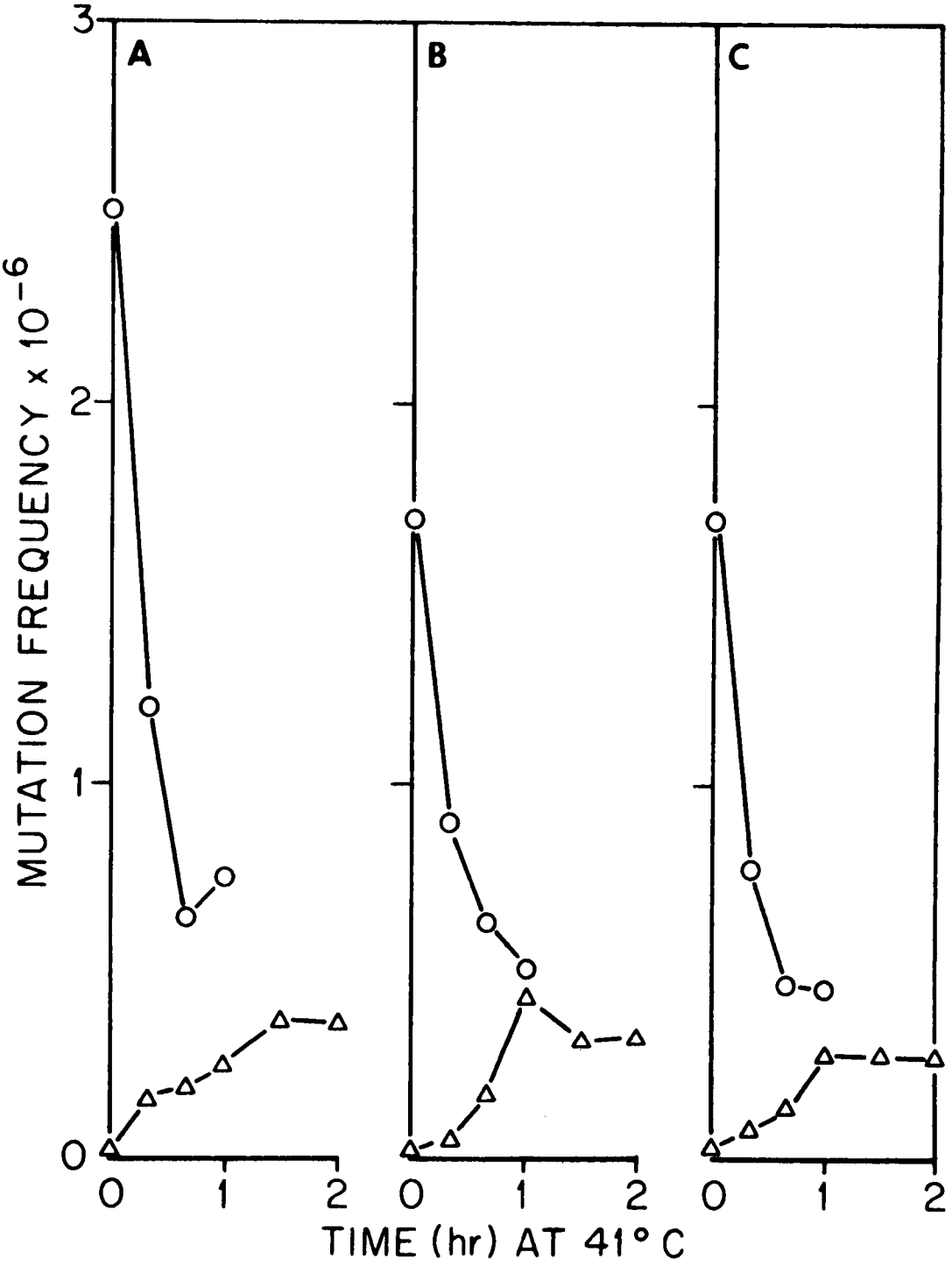


Figure 19

defect in a step in excision that occurs after action of the UV-endonuclease[111]. Figure 19B shows that mutation fixation at 41° C and repair of premutational lesions also occur in strain dnaQ9 uvr2.

The UV-sensitive H. influenzae strain DB115 can excise UV-induced pyrimidine dimers but is apparently slow to rejoin the gaps formed in the DNA by the excision enzymes[114]. As seen in Figure 19C, this strain is also able to repair premutational lesions and perform the nonreplicative type of mutation fixation.

IV. DISCUSSION

Cellular Processes in Mutagenesis: Information Obtained with the Haemophilus System

Results presented in Figures 2 (p. 15) and 4 (p. 20) indicate that most of the mutation fixation observed after treatment of wild-type H. influenzae with NC requires DNA replication. As will be discussed later, the actual change in the nucleotide sequence in the DNA could occur either during or after replication of the DNA containing mutagen-induced lesions.

As discussed earlier (Figures 5, p. 23, and 6, p. 26), mutation fixation in the majority of cells continues throughout nearly half of the first round of DNA replication after treatment. Since the bulk of the mutation fixation requires DNA replication, premutational lesions are not confined to regions near the replication forks. Cerdà-Olmedo et al.[21] demonstrated preferential MNNG-induced mutagenesis at the replication fork in Escherichia coli, and concluded that MNNG reacts to form premutational lesions preferentially at the replication fork. If premutational lesions in NC-treated H. influenzae were present only in the region of the replication fork, then mutation fixation would be expected to increase rapidly and level off very soon after the mutagenic treatment, unless mutation fixation involved replication through lesions near the replication fork followed by a repair process that continued for considerably longer. However, this possibility is unlikely since

if treated dna9 cells are incubated at 41° C after completion of about 10% of one round of replication, mutation fixation levels off at a considerably lower level than if DNA replication is allowed to proceed (Figure 4, p. 20). Therefore, even if postreplication repair is involved in the replication-dependent mutation fixation, the lesions involved are not located solely at or near the replication fork.

As stated previously[51, 58], the data of Cerdà-Olmedo et al.[21] could easily be explained by an increased probability of repair for premutational lesions located away from the replication fork. If repair of premutational lesions occurred, mutation frequency would be highest at loci that were about to be replicated at the time of mutagen treatment, since premutational lesions far away from the replication fork would have a greater chance of being repaired before the replication fork reached them. Repair of premutational lesions distant from the replication fork may also be responsible for the observation that in the vast majority of cells mutation fixation leveled off after completion of only 35-53% of the first round of replication after treatment with NC (Figure 5, p. 23).

Not all of the mutation fixation in NC-treated cells requires DNA replication. About 20-30% of the mutation fixation occurs by some other process, most probably misincorporation during repair of DNA containing mutagen-induced lesions. The involvement of the recl gene product in the nonreplicative type of mutation fixation is ruled out, since strain dna9 recl accomplishes at least as much mutation fixation at 41° as does strain dna9 (Figure 11, p. 40). Furthermore, the error-prone mechanism responsible for the nonreplicative mutation fixation is

not the same mechanism that removes UV-induced pyrimidine dimers from the DNA, since mutants deficient in three different stages of excision repair all performed mutation fixation in the absence of DNA replication (Figure 19, p. 62).

Besides having a positive effect through the production of mutations, repair could also have a negative effect on mutagenesis by removal of premutational lesions before mutation fixation occurs. A decrease in the final mutation yield was observed in H. influenzae when DNA replication was prevented for a time after treatment with NC before mutation fixation was allowed to proceed (Figure 8, p. 32). The most reasonable interpretation of this result is that premutational lesions are repaired during incubation of the cells in the absence of DNA replication. The presence of a fraction of mutations remaining after the repair that occurs in the absence of DNA replication is consistent with the finding that 20-30% of the mutation fixation is independent of DNA replication. The premutational lesions that are "fixed" by a nonreplicative process ought to be converted to a nonrepairable form by the fixation process.

Repair of premutational lesions must occur by recognition and removal from the DNA of lesions that are capable of inducing replication-dependent mutation fixation. The mechanism that removes these lesions could be error-prone, in which case part or all of the nonreplicative mutation fixation could occur during the prereplication repair of premutational lesions. Alternatively, the mechanism that is capable of removing premutational lesions could be error-free, and a different

process, acting on either these same lesions or on other type(s) of damage, could be the error-prone repair process that leads to the non-replicative mutation fixation.

One way to determine whether repair of premutational lesions and nonreplicative mutation fixation occur by the same mechanism involves a search for mutant strains that are deficient in one or both of these phenomena. Such a search has begun, and entails the construction of strains carrying the dna9 mutation along with various mutations that cause defects in repair and recombination. So far the dna9 mutation has been combined with the rec1 mutation and with three mutations that affect different steps in repair of UV-induced pyrimidine dimers (uvr1, uvr2 and DB115). The dna9 rec1 mutant shows little or no detectable repair of premutational lesions. This result was expected, since the detection of repair of premutational lesion depends on the formation of mutations by a replication-dependent process, and all of the mutation in the rec1 strain appears to arise by nonreplicative mutation fixation. The observation that repair of premutational lesions occurs in all three strains carrying the mutations affecting excision-repair of UV-induced pyrimidine dimers (Figure 19, p. 62) indicates that repair of premutational lesions does not occur by the dimer-excising mechanism.

The possibility must be considered that repair of premutational lesions is an artifact. For example, the result that was interpreted in terms of repair of premutational lesions could have been caused by preferential death of potential novobiocin-resistant mutants during the incubation of dna9 at 41° C. This seems unlikely, for two reasons.

First, although a decrease in viability usually occurs when dna9 is held at 41° C, the extent of lethality is quite variable from one experiment to the next, while the extent of reduction in the novobiocin-resistant mutations resulting from incubation at 41° C is not variable. Secondly, it is difficult to imagine how the lethality resulting from incubation of strain dna9 at the restrictive temperature would occur preferentially in the cells destined to become resistant to novobiocin. A second possible source of such an artifact would be the formation of premutational lesions that are chemically unstable. Although nobody knows what the premutational lesions are, if alkylated bases are mutagenic, then depurination could lead to loss of mutations in the absence of fixation. However, the known depurination reactions occur much more slowly than the observed disappearance of mutations that results from incubation of mutagen-treated dna9 at 41° C[17, 64, 69].

The conclusions that most mutation fixation in NC-treated H. influenzae requires DNA replication and that premutational lesions can be repaired before replication takes place are similar to conclusions drawn from work with Paramecium aurelia. Kimball[51] found that the highest mutation frequency was obtained when the cells were treated with MNNG just before the onset of DNA replication (late G_1). The earlier in G_1 the cells were treated, the lower the mutation yield. Treatment during G_2 and mitosis yielded very few if any mutants. It was proposed that MNNG induces repairable premutational lesions that are converted to final mutations during DNA replication. Similar conclusions were made from earlier experiments with UV and ionizing

radiation and di- and trifunctional alkylating agents[48, 49, 50, 52, 53, 55, 56].

It appears from the data of Figure 12 (p. 43) that replication-associated mutation fixation occurs in the absence of the recl gene product. However, the mutations that are fixed by this process in the recl strain are unstable, and disappear at later times, leaving only the mutations fixed by a nonreplicative process. The reason for this disappearance is not clear.

One possible explanation for the disappearance of newly fixed mutations in the recl strain is that although mutations may be fixed by misincorporation opposite certain mutagen-induced lesions during replication, in the recl strain this newly synthesized DNA is degraded. In support of this possibility, degradation of DNA synthesized immediately after treatment of the recl strain with NC occurred more extensively than in wild-type cells (Figure 16, p. 54).

If degradation is responsible for the disappearance of newly fixed mutations in the recl strain, it must be associated with some process that does not begin immediately after fixation and does not require subsequent DNA replication (see Figures 12, p. 43, and 13, p. 46). Perhaps the newly fixed mutations are in an intermediate state that is later converted to a final form in wild-type cells. In recl cells the conversion of newly fixed mutations to a final form may be lacking, and the DNA containing the newly fixed mutations may be subsequently degraded. Evidence that newly fixed mutations are not in their final form, even in wild-type cells, comes from experiments that showed that newly fixed mutations (in lysates made from MNNG-treated cells)

are very sensitive to inactivation by 313-nm irradiation, whereas incubation for longer times converts them to a final form that does not exhibit extreme sensitivity to inactivation by 313-nm irradiation[58].

The newly synthesized DNA in both wild-type and recl mutagen-treated cultures was smaller than prelabeled DNA of untreated cells. In the wild-type treated cells the molecular weight of the newly synthesized DNA rapidly increased upon incubation in growth medium, becoming the same as the DNA of untreated cells by 20 minutes. In the recl cells, however, there was a much slower increase in molecular weight of the DNA synthesized immediately after treatment. There is considerable evidence that during replication DNA is synthesized discontinuously in short fragments that are subsequently joined together to form high molecular weight DNA[95, 96, 97, 122, 123]. Previous experiments (J. E. LeClerc, unpublished data) suggest that the recl strain is not slow in the normal joining of the "Okazaki fragments". Furthermore, if the recl strain were slow in joining Okazaki fragments, the sedimentation profiles of DNA labeled for 15 minutes after treatment of recl cells with NC (Figures 17, p. 56, and 18, p. 59) should show considerable amounts of slower sedimenting DNA, rather than closely resembling the profiles of DNA from wild-type cells. Therefore, since the discontinuities present in newly synthesized DNA strands of NC-treated recl cells are repaired much more slowly than in rec$^+$ cells, they must be distinct from the type of strand interruption transiently present in untreated cells.

A similar phenomenon, known as postreplication repair, has been observed in UV-irradiated E. coli and interpreted in the following way.

Newly synthesized DNA in UV-irradiated cells contains gaps, probably opposite pyrimidine dimers[105, 118]. The low molecular weight DNA synthesized after UV irradiation is later joined up[105] by a process that requires a functional recA gene product[118]. This process involves genetic exchange between DNA synthesized before and after UV irradiation, and the number of exchanges roughly equals the number of pyrimidine dimers present in the DNA[106]. Postreplication repair in UV-irradiated H. influenzae was previously shown to be deficient in the recl mutant[75] but normal in another recombination-deficient mutant, rec2, which shows normal MNNG-induced mutagenesis (R. F. Kimball, personal communication). As in the work with UV-irradiated H. influenzae, it is not clear whether the strand joining in NC-treated recl is inhibited by excessive breakdown of the newly synthesized DNA or whether the breakdown results from a deficiency in the joining of gaps in newly synthesized DNA.

Perhaps the error-prone step in the replication-dependent mutation fixation does not occur during replication, but occurs during the joining of discontinuities present in newly synthesized DNA of NC-treated cells. This strand joining could occur in either of two ways. First, a repair mechanism may exist that directly fills in the gaps present in the newly synthesized DNA. The recl strain may be able to perform the repair synthesis step but unable to complete the strand joining. Consequently, stretches of DNA synthesized during early stages in the repair (and containing newly fixed mutations) might be later degraded in recl cells.

Alternatively, the strand joining might be accomplished by a recombinational repair mechanism such as that proposed for UV-irradiated

E. coli. The recl strain is deficient in a late step in recombination, since covalent linkage between donor and recipient DNA occurs during transformation of this mutant[77, 94]. The recl strain may likewise be able to carry out sufficient steps in a postreplication recombinational repair process to allow error-prone repair synthesis to occur. This intermediate stage in the process would therefore be reached in both wild-type and recl cells. In wild-type cells the intermediate would be converted to the final repaired product but in recl cells the intermediate would accumulate and, in the absence of completion of repair, might be later degraded. In any case, some slow strand joining may occur in recl cells by an alternate, error-free mechanism.

In support of the idea that the error-prone step in replication-dependent mutation fixation occurs during postreplication repair, it is presently believed, as suggested by Bridges et al.[9] and Bridges and Munson[15] and later proposed by Witkin[145], that certain UV-induced mutations in E. coli arise during error-prone postreplication repair. This hypothesis is supported by the finding that E. coli strains carrying the recA mutation (which eliminates postreplication repair) show no detectable UV-induced mutagenesis[88, 144], whereas the frequency of UV-induced mutation is greatly increased in excision-defective strains[43, 141, 143] in which survival apparently depends on post-replication repair. As in the case of NC-induced mutagenesis in Haemophilus, however, the possibility still exists that in E. coli mis-incorporation occurs during replication of DNA containing UV-induced lesions, and in the absence of a functional recA gene product, the newly synthesized

DNA (containing the mutations) is degraded. DNA synthesized shortly after UV-irradiation of E. coli recA cells is in fact degraded excessively[23]. This degradiation appears to be caused by action of the ATP-dependent nuclease (exonuclease V) coded for by genes recB and recC[136]. The recA and exrA (lex) genes are apparently involved in the inhibition of exonuclease V in irradiated cells[86, 89, 136]. If degradation (by exonuclease V) of newly established mutations in UV-irradiated E. coli occurs, then recA recB and recA recC strains of E. coli should exhibit UV-induced mutagenesis. This experiment has not yet been reported.

Cellular Processes in Mutagenesis: Information Obtained with Other Systems

Probably the most thoroughly investigated type of mutagenesis has been UV-induced mutagenesis. A large portion of the work has utilized the E. coli system. The results indicate that UV-induced mutagenesis in E. coli is a complex phenomenon, involving a number of cellular processes. Complicating aspects exist, such as the dependence of mutagenic mechanism on the strain being used and on the type of mutation being detected. By ignoring certain contradictions and accepting some speculation it is possible to contrive the following general account of UV-induced mutagenesis in E. coli, certain features of which resemble mutagenesis in H. influenzae induced by N-nitroso compounds.

It appears that in wild-type E. coli cells the first step in mutation to streptomycin resistance is a repair process that occurs

before DNA replication[12, 92]. This conclusion is based on a method of measuring "mutation fixation" completely different from that used in the Haemophilus system. The method is based on the fact that UV-induced mutations can be eliminated by enzymatic photoreactivation immediately after UV irradiation. In other words, pyrimidine dimers induced by the irradiation, which are probably the premutational lesions for most if not all UV-induced streptomycin-resistant mutations[146], are split by the photoreactivating enzyme when the cells are irradiated with visible light immediately after UV irradiation. However, the first step in conversion of the pyrimidine dimers to the final mutation "fixes" the premutational damage in a form that is no longer subject to photoreactivation. It was found that loss of photoreversibility occurred after UV irradiation under conditions that prevented DNA replication and protein synthesis but allowed excision of pyrimidine dimers. Loss of photoreversibility of mutation to streptomycin resistance was blocked by dinitrophenol, which prevents excision of pyrimidine dimers[115]. The results were interpreted as indicating that the first step in the majority of the UV-induced mutagenesis to streptomycin resistance is excision of pyrimidine dimers, and that repair synthesis, required for filling in of the gap left by excision, is error-prone. It is not certain whether error-prone repair of gaps left by excision occurs before or after replication. Nishioka and Doudney[92] found that even after loss of photoreversibility of mutation to streptomycin resistance had occurred, the mutations could not be expressed until DNA replication was allowed to occur (by adding thymine back to the medium). They proposed that

semiconservative DNA replication is a part of the mutational process. They did not consider the possibility that thymine deprivation prevents prereplicative repair-type DNA synthesis.

Reports that recA[27] and exrA[151] genes play roles in excision-repair of UV-induced pyrimidine dimers are consistent with the lack of UV-induced mutagenesis in recA[88, 144] and exrA[143] strains. The products of genes polA and exrA are apparently involved in different mechanisms for filling in of gaps made by excision of pyrimidine dimers[152]. The resynthesis step of excision repair produces both small and large patches of resynthesized DNA[26]. Since there is an increased amount of long-patch repair synthesis in strains (polA) lacking DNA polymerase I activity and no long-patch repair in a recA recB double mutant, Cooper and Hanawalt[27] proposed that short-patch repair synthesis is performed by DNA polymerase I, whereas long-patch repair synthesis is dependent on functional recA and recB gene products. It is likely that the long-patch repair synthesis is also controlled by the exrA gene product[152]. It is unlikely that the long repair patches arose via postreplication recombination repair, since no repair synthesis was detected in a uvrA strain[25], which does not excise pyrimidine dimers but does perform post-replication repair.

The error-prone process in UV-induced mutation to streptomycin resistance may be the long-patch repair synthesis controlled by the recA, recB and exrA genes. Although there is evidence that this type of repair synthesis is dependent on DNA polymerase III activity[153], work with toluenized E. coli[87] indicates that DNA polymerase II is also capable of synthesis resembling the long-patch repair observed in vivo.

UV-induced mutation to streptomycin resistance in strains of E. coli deficient in excision of pyrimidine dimers is associated with DNA replication. Nishioka and Doudney[92] found that loss of photoreversibility of mutation to streptomycin resistance in an excision-defective strain is dependent on both DNA replication and protein synthesis. The same result was obtained with UV-induced reversion to prototrophy (trp^- to trp^+)[15, 92]. Thus, in excision-defective E. coli cells UV-induced mutagenesis occurs by a replication-dependent process, analogous to most NC-induced mutagenesis in H. influenzae. As mentioned before, the current popular hypothesis is that these mutations arise by an error-prone postreplication repair mechanism. As in the case of gap filling after excision of pyrimidine dimers, the resynthesis step during post-replication repair in UV-irradiated E. coli can apparently be carried out by either DNA polymerase I or DNA polymerase III[108].

UV-induced mutation to prototrophy in wild-type E. coli appears to occur by two mechanisms. Some of the revertants (probably the true revertant) apparently arise by an excision-repair mechanism, like streptomycin-resistant mutants, but the majority of the revertants (suppressor mutants) arise by the replication-dependent mechanism, even in uvr^+ cells[92, 93]. UV-induced suppressor mutants in uvr^+ cells are different from other types of UV-induced mutants in several respects. First, these mutants are not reversible by enzymatic photoreactivation, although they are photoreversible[139, 140, 146], probably by an indirect photoprotective effect on repair of the premutational lesions. The effect of the photoreversing light can be abolished by subsequent incubation with inhibitors of dark repair, such as caffeine and acriflavine[139]. Thus, the

premutational lesions for UV-induced suppressor-type revertants in uvr$^+$ cells may not be pyrimidine dimers, since they are not removed by enzymatic photoreactivation. Evidence which strongly supports this view comes from work by Setlow and Boling[110]. They found that in wild-type E. coli cells the action spectrum for reversion to prototrophy resembled the UV absorbance spectrum for purine residues, not that of thymine. On the other hand, in cells in which pyrimidine dimer excision was absent because of a mutation or because of the presence of acriflavine, the action spectrum for reversion to prototrophy resembled that for cellular inactivation, which is caused by pyrimidine dimers[109].

The premutational damage leading to the suppressor-type revertants to prototrophy in uvr$^+$ cells, unlike that for other types of mutation[9, 147], is susceptible to a phenomenon called "mutation frequency decline" (MFD), which is the sharp reduction in mutation yield resulting from incubation of cells in the absence of protein synthesis immediately after UV irradiation[30, 137]. MFD of suppressor mutations is rapid, being finished within about 20 minutes after irradiation, and is inhibited by acriflavine[138]. In accordance with the view that the premutational damage leading to suppressor-type revertants to prototrophy is not pyrimidine dimers, MFD is not accompanied by an increase in survival[137] and occurs under conditions disfavoring dimer excision, whereas dimer excision occurs under conditions preventing MFD[142]. MFD therefore appears to result from removal from the DNA of a type of non-dimer UV-induced lesion that is capable of producing suppressor mutation in uvr$^+$ cells. The mechanism for removal of these lesions is probably not the same as that for excising

pyrimidine dimers. The MFD mechanism is likely to be involved in the indirect photoreversal of prototrophic mutations in wild-type cells[146].

Other repair mechanisms may exist which are capable of removing UV-induced premutational lesions. For example, under the same conditions used to demonstrate MFD, reduction in the yield of streptomycin-resistant mutants also occurs[24, 148]. This process is different from true MFD, however, since it occurs only at low doses of UV irradiation, whereas MFD occurs at all doses, and it shows drastically different kinetics than MFD (much slower and beginning after MFD is completed)[148]. It was proposed that this type of reduction in mutation yield may reflect an increased probability of filling in excision gaps by the error-free mechanism rather than the error-prone mechanism[148].

Although these phenomena may be quite different from the disappearance of premutational lesions observed in H. influenzae and Paramecium aurelia, they nevertheless serve as indications that mechanisms exist for removal of premutational lesions.

Much less is known about ionizing radiation-induced mutation than UV-induced mutagenesis, but work with E. coli suggests that some cellular processes are involved in production of both kinds of mutation. For example, similar to the case of UV-induced mutation, there is very little gamma radiation-induced mutagenesis in recA and exrA strains of E. coli[11, 61, 107].

Indirect evidence suggests that ionizing radiation-induced mutagenesis in E. coli occurs during the repair of radiation-induced strand breaks. At least two mechanisms exist for repair of ionizing radiation-induced strand breaks in E. coli. About 90% of the breaks

are repaired by "type II" repair, which is completed in buffer in a few minutes at 37° C and is dependent on DNA polymerase I activity[129, 130]. About 10% of the induced breaks are repaired by a "type III" growth medium-dependent process that is completed in 20-40 minutes at 37° C and requires functional recA, recB and exrA genes[47, 84, 107]. A mutant carrying both polA1 and exrA genes performs neither type II nor type III repair of ionizing radiation-induced breaks[151]. Type III repair is also dependent on DNA polymerase III activity[150] and is abolished in the temperature sensitive DNA synthesis mutant dnaB at the restrictive temperature[34].

The polA1 mutation causes elevated ionizing radiation-induced mutagenesis[13]. It has been proposed that this increased mutability is due to increased error-prone type III repair in the absence of type II repair of radiation-induced single strand breaks[13]. It is not certain whether type III repair occurs before, during or after passage of the replication fork. The fact that type III repair does not occur when DNA synthesis is stopped by elevated temperature in either the dnaB or dnaE mutants suggests that this may be a postreplication repair process similar to that which occurs after UV irradiation. In the case of gamma radiation-induced mutagenesis in bacteriophage T4 it has been shown that T4 DNA replication is required for establishment of most mutations[10].

Another possible mechanism for ionizing radiation-induced mutagenesis is error-prone filling in of gaps after excision of radiation-induced lesions from the DNA. Such a mutagenic mechanism has not yet been demonstrated, but an endonucleolytic activity acting on gamma-irradiated as well as UV-irradiated DNA has been demonstrated in

Micrococcus luteus[101, 116]. Evidence for excision of the gamma ray-induced endonuclease-sensitive sites from DNA of E. coli has been obtained[101], and evidence for excision of radiolysis products has been obtained in Micrococcus radiodurans[38, 39].

From the results concerning UV- and ionizing radiation-induced mutagenesis in E. coli it appears that the same repair processes are involved in mutagenesis induced in this organism by a variety of mutagenic agents. This emerging view is strengthened by recent work on mutagenesis induced in E. coli by the reactive arylalkylating carcinogen, 7-bromomethylbenz(a)anthracene. Reaction of this chemical with DNA in aqueous solution leads to the substitution of benz(a)anthracenyl-7-methyl residues on the extranuclear amino groups of guanine, adenine and cytosine[29]. This compound has been tested for induction of prototrophic revertants in E. coli WP2 and some of its repair-deficient derivatives[127]. No mutation was observed in the exrA and uvrA exrA strains, while at low doses mutation induction was greatly elevated in the uvrA strain, compared with wild-type. Results with rec$^-$ strains have not yet been reported. Thus, mutagenesis of E. coli with 7-bromomethylbenz(a)anthracene requires a functional exrA gene product, as with UV- and ionizing radiation-induced mutagenesis. Furthermore, it appears that premutational lesions formed by the arylalkylating agent are efficiently removed from the DNA of wild-type cells by the same mechanism that excises UV-induced pyrimidine dimers. This conclusion is supported by the finding that selective in vivo excision of arylalkylated products from the DNA has been demonstrated[131]. There was no detectable excision in the uvrA exrA strain and the amount of excision in the uvrA strain was greatly reduced

from that in wild-type cells. Maher et al.[85] obtained evidence for the excision of 7-bromomethylbenz(a)anthracene-induced lesions from DNA in B. subtilis by the pyrimidine dimer-excising mechanism. Transforming DNA treated with this reagent showed lower survival and produced fewer mutations via transformation when the recipient strain was defective in excision of pyrimidine dimers. Furthermore, evidence was presented that a UV endonuclease purified from rat liver introduced single-strand nicks into DNA in proportion to the amount of hydrocarbon bound.

Similar results were obtained in the induction of prototrophic revertants in E. coli by the carcinogen 4-nitroquinoline oxide (4NQO)[62]. With this mutagen no detectable reversion occurred in a recA strain, while the reversion rate was greatly enhanced in a uvrA strain. The data were interpreted to mean that 4NQO-induced premutational lesions are repairable by the pyrimidine dimer-excising mechanism and produce mutations by the same mechanism as UV irradiation.

Mutagenesis induced by various monofunctional alkylating agents has recently been studied in wild-type and various repair-deficient strains of E. coli. There are conflicting reports as to the effect of the pyrimidine dimer-excising mechanism on mutagenesis induced by monofunctional alkylating agents. Kondo et al.[62] reported that in excision-defective E. coli mutagenesis induced by methyl methanesulfonate (MMS), ethyl methanesulfonate (EMS) and MNNG did not differ appreciably from that in wild-type cells. Bridges et al.[14] obtained the same result with MMS. Hince and Neale[45], however, studying mutagenesis induced by N-nitroso-N-methylurea (NMU), found that at all but the lowest doses of mutagen the mutation frequency in the uvrA strain was only about half that in the

wild-type strain. In H. influenzae Kimball et al.[59] found that one excision-defective strain (DB112, carrying the uvr1 mutation) showed only about half as much MNNG-induced mutation as the wild-type strain. These differences are not drastic, though, and their significance is not yet clear.

In recA strains of E. coli no mutagenesis has been observed after treatment with MMS[14, 62], whereas little if any reduction in mutability occurred with EMS[62]. Hince and Neale[45] found that NMU-induced mutagenesis was slightly elevated in a recA strain, compared to that in wild-type cells. Kondo et al.[62] reported that MNNG-induced mutagenesis was about three to four-fold lower in a recA strain than in wild-type. This figure is similar to the reduction in NC-induced mutagenesis in H. influenzae strain rec1 (see Figure 10, p. 37). The exrA strain showed no detectable mutagenesis with MMS[14], while with MNU there was little difference in mutagenesis between exrA and wild-type strains[45].

In strains of E. coli deficient in DNA polymerase I (polA) Kondo et al.[62] and Bridges et al.[14] reported that MMS-induced mutagenesis was elevated about three-fold, compared to that in wild-type cells. Bridges et al.[14] proposed that this effect may be caused by increased error-prone repair of MMS-induced single strand breaks in the absence of DNA polymerase I-catalyzed strand rejoining. The same hypothesis was previously used to explain elevated gamma ray-induced mutagenesis in a polA strain[13]. Contrary to the data of Kondo et al.[62] and Bridges et al.[14] (which involved reversion to prototrophy), Smirnov et al.[117] reported that the polA1 mutation has no effect on MMS-induced mutation to streptomycin resistance. The difference may be due to different mutagenic

pathways for production of streptomycin-resistant mutants and prototrophic revertants. This is not unlikely, since MMS-induced mutagenesis seems to resemble UV-induced mutagenesis, in which mutagenic pathways (in *uvr*$^+$ strains) are different for streptomycin-resistant mutants and prototrophic revertants[92, 93]. Kondo *et al.*[62] reported that unlike the case with MMS, absence of DNA polymerase I activity had little or no effect on EMS- and MNNG-induced reversion to prototrophy in *E. coli*.

From the results in *E. coli* it appears that not all monofunctional alkylating agents induce mutation by the same mechanism. A possible explanation for this is that monofunctional alkylating agents react with DNA to form a large number of products, and the spectrum of products depends in part on the type of alkylating agent used. It would therefore be useful to review what is known about reactions between monofunctional alkylating agents and DNA, and to speculate as to how the various products might interact with cellular processes to produce mutations.

Reactions Between Monofunctional Alkylating Agents and DNA and Possible Molecular Consequences

Treatment of DNA with an alkylating agent results in reactions at various sites on the DNA molecule, and formation of a variety of products. The reactions proceed by nucleophilic substitution[103]:

$$\text{DNA}\text{—}\text{X} + \text{Y}\text{—}\text{R} \longrightarrow \text{DNA}\text{—}\overset{\oplus}{\text{X}}\text{—}\text{R} + \text{Y}^{\ominus},$$

where X is a nucleophilic group in DNA and Y is an electrophilic group on the alkylating agent. When Y departs from the alkylating agent, it

carries with it the pair of electrons that bound it to the carbon atom in R. The displacing nucleophilic group in DNA then provides an electron pair during formation of the alkylated product. The reaction may proceed through first-order (SN1) or second-order (SN2) nucleophilic substitution[46, 99, 104]. The SN1 mechanism involves unimolecular formation of a carbonium ion by the alkylating agent (rate-limiting step), followed by rapid reaction with the nucleophile:

$$YR \rightleftharpoons Y^{\ominus} + R^{\oplus}$$
$$-X + R^{\oplus} \longrightarrow -X\overset{\oplus}{R}$$

The SN2 mechanism proceeds through a transition state complex which dissociates into the products:

$$-X + R-Y \longrightarrow [-\overline{X}^{\delta}\cdots\overset{\oplus}{R}\cdots\overline{Y}^{\delta}] \longrightarrow -X-\overset{\oplus}{R} + Y^{\ominus}$$

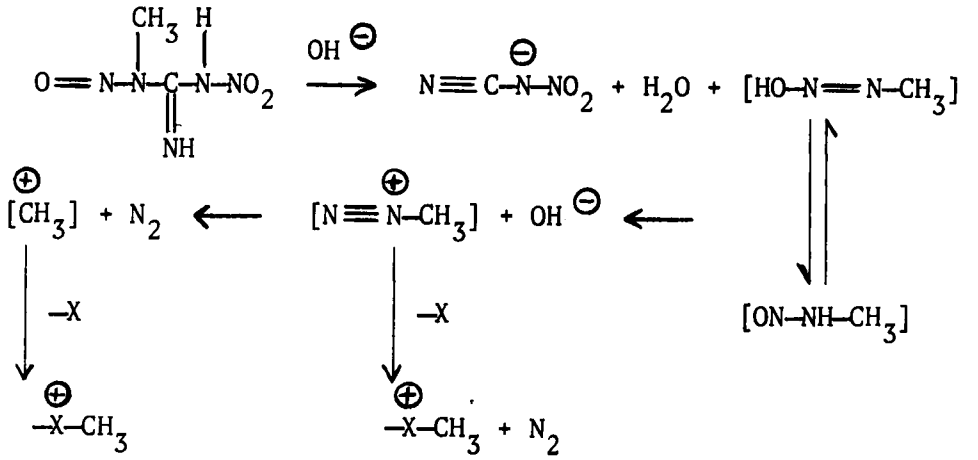
Nucleophiles in the medium such as water molecules and certain anions can compete with nucleophilic sites on DNA in the reaction with alkylating agents[99]. Although the SN2 mechanism predominates in most alkylation reactions, some degree of SN1 mechanism often operates simultaneously, the extent depending on the type of reagent and conditions of reaction[66, 99].

In reactions of alkylating agents with DNA, the most reactive site is the most nucleophilic site, N-7 of guanine[65]. Less reactive sites of alkylation include N-3, N-1 and N-7 of adenine, N-3 of cytosine, O-6 and N-3 of guanine, and N-3 and O-4 of thymine[66, 71, 72, 74]. SN2-type reagents react bimolecularly with nucleophilic sites, the extent of reaction depending on the nucleophilicity of the site. SN1-type

reagents, however, react more readily with weakly nucleophilic sites than SN2-type reagents. Alkylation of 0-6 of guanine (a weakly nucleophilic site) occurs with reagents such as N-nitroso compounds and EMS that can react at least partially through the SN1 mechanism, whereas greatly reduced amounts of O^6-methylguanine are formed by SN2-type reagents such as MMS and dimethyl sulfate[73, 74]. N-methyl-N-nitrosourea has recently been shown to react with DNA to form small amounts of O^4-methylthymine[72]. The phosphate groups in DNA react to form phosphotriesters[2, 67]. This reaction seems to occur more readily with SN1-type reagents than with SN2-type reagents[2, 74]. In general, SN1-type reagents attack a wider spectrum of sites on DNA and appear to be more potent carcinogens and more mutagenic in some test systems than SN2-type compounds[67, 81, 82, 125].

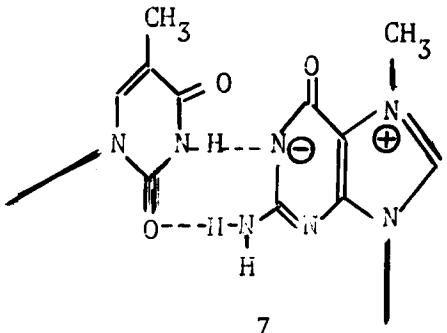
The N-nitroso compounds ($O{=}N{-}N\langle^{R}_{R'}$) include some of the most potent mutagens and carcinogens known. Dialkyl nitrosamines are stable, and unreactive toward DNA *in vitro*, but are enzymatically activated in eucaryotic cells (for example, by oxidative removal of one alkyl group)[66]. Compounds having electrophilic R' (such as MNNG) are very reactive. It has been widely assumed that alkylation of nucleic acids by active N-nitroso compounds involves the intermediate formation of diazoalkanes (which are formed from N-nitroso compounds in alkali). This idea has been discredited by the findings of Lijinsky *et al.*[78], demonstrating by mass spectrometry that with deuterated dimethylnitrosamine administered to rats the methyl group is transferred intact to the N-7 position of guanine. A similar result was obtained for

alkylation of E. coli DNA in vitro and in vivo by deuterated MNNG[124]. It has been proposed[66, 74] that methylation of DNA by MNNG occurs by the following reaction:

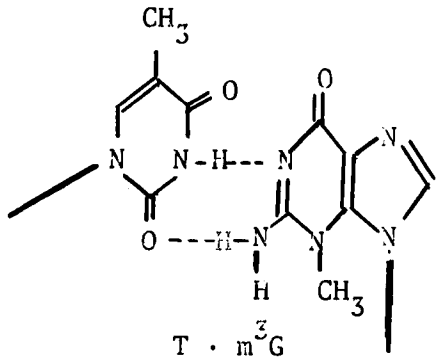


The alkali-catalyzed decomposition of MNNG predominates at pH values above about 4.5. MNNG is attacked by hydroxide ion or buffer anion to form the nitrocyanamide ion which has been isolated and identified. The product methyldiazohydroxide or its isomer monomethylnitrosamine then ionizes to form the methyldiazonium ion, which may react directly with a nucleophilic site on DNA or may decompose to methylcarbonium ion and nitrogen before reaction with DNA.

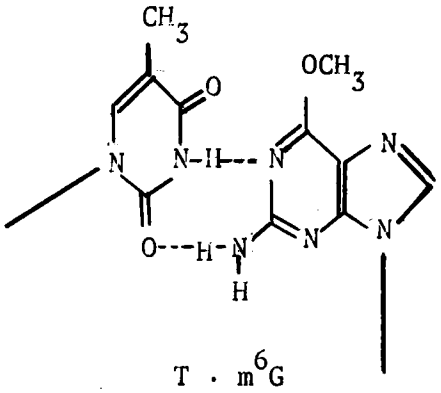
One possible mechanism of mutagenesis involves atypical base pairing during DNA replication[66, 135]. The following base-pairing schemes have been proposed, showing atypical pairing with alkylated bases.



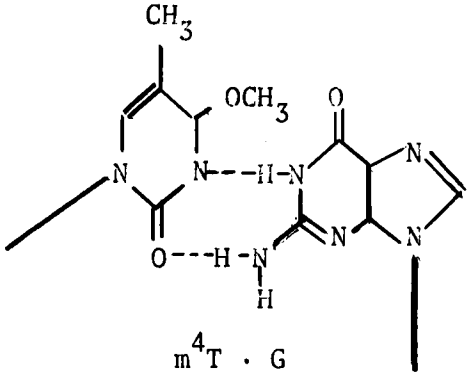
T · m^7G



T · m^3G



T · m^6G



m^4T · G

Pairing between ionized 7-methylguanine and thymine was proposed by Lawley and Brooks[68]. This scheme seems very unlikely, for several reasons. First, 7-methylguanine in polynucleotides (unlike the monomer) is not ionized at physiological pH[40]. Also, no evidence for mispairing of 7-methylguanine was obtained in incorporation studies using RNA[83] and DNA[40] templates. Finally, all monofunctional alkylating agents react with DNA to form 7-methylguanine as the major product, whereas there is a very wide variance in their mutagenicity.

Discovery of 3-methylguanine as a very minor alkylation product in DNA[71] led to the suggestion that it might mispair with thymine. In the base-pairing scheme shown, 3-methylguanine is in the amino form. According to Lawley[66] it is not known whether this base would exist in

the amino form or the imino form, which should exhibit normal base pairing.

Base-pairing between $\underline{O}^6$-methylguanine and thymine has been postulated to be responsible for mutagenesis and carcinogenesis following exposure to SN1-type reagents[28, 81, 82]. O-alkylation forces the guanine residue into the tautomeric configuration that was proposed for ionized 7-methylguanine, allowing guanine-thymine pairing without distortion of the overall dimensions of the base pairs[72]. Support for this type of anomolous base-pairing comes from the demonstration that when a copolymer of cytidylic and $\underline{O}^6$-methylguanylic acids was used as template for RNA polymerase, misincorporation of UMP occurred[37].

The possibility of mispairing of alkylated guanine residues with thymine receives further support from studies of the induction of revertants in bacteriophage. Krieg[63] found that most T4 rII revertants induced by EMS are G·C → A·T transitions. Similarly, Tessman et al.[128] reported that the most frequent type of EMS-induced reversion in bacteriophage S13 and ØX174 involved G·C → A·T transition. When all the available alkylating agent-induced base change data are reviewed it appears that G·C → A·T transitions are generally the most common base changes induced by these compounds, although several cases of A·T → G·C transitions exist[31].

Formation of small amounts of $\underline{O}^4$-methylthymine in DNA treated with N-methyl-N-nitrosourea has recently been detected[72]. This product was not previously detected because it is extremely acid-labile and was converted to thymine during earlier procedures for degrading DNA.

It was proposed that O^4-methylthymine could base-pair with guanine, leading to A·T → G·C transitions.

The likelihood of atypical base pairing during replication of DNA containing alkylated bases as well as the probability of misincorporation during repair processes, probably depend on the properties of the individual DNA polymerases involved. The two DNA polymerases that have been most thoroughly characterized, T4 DNA polymerase and DNA polymerase I of E. coli, both possess 3' to 5' exonucleolytic activities that efficiently remove mismatched bases after their misincorporation during DNA synthesis[18, 42]. Muzyczka et al.[90] found that in bacteriophage T4 the 3' to 5' exonucleolytic activity was reduced in DNA polymerase from mutator strains and increased in DNA polymerase from antimutator strains. Hershfield[41] found that the defect in one particular T4 mutator DNA polymerase consisted of increased misincorporation, rather than decreased removal of mismatched incorporated bases. Thus, the fidelity of DNA synthesis depends on proper base selection as well as the efficiency of "proofreading". Differences in the types of base changes induced by a given mutagen in different systems (bacteriophages S13 and T4, for example)[1] could be due to differences in the specificities of the base selection and proofreading functions of the polymerases involved.

Considerable evidence has accumulated, both in H. influenzae and in E. coli, indicating that repair processes are important in mutagenesis. It would therefore be worthwhile to discuss the available evidence for repair of alkylation-induced damage in bacteria.

The data of Figure 15 (p. 51) indicate that single strand breaks induced by treatment of H. influenzae with NC are repaired. Repair of MNNG-induced strand breaks was previously demonstrated in H. influenzae[54] and similar repair phenomena have been shown to occur in MMS-treated Bacillus subtilis[102], in MMS-treated bacteriophage lambda DNA[7], and in MNNG-treated E. coli[98].

The origin of the breaks detected by alkaline sucrose gradient centrifugation is not known. It is unlikely that they result from hydrolysis of alkylation-induced phosphotriesters, because phosphate triesters have been shown to be very stable[2]. The strand breaks could be formed in the DNA by endonucleolytic attack of the alkylated DNA before lysis of the cells on top of the alkaline sucrose gradients. An endonuclease has been isolated from Micrococcus lysodeicticus which is capable of introducing single strand breaks at or near alkylated bases in DNA lacking depurinated sites[121]. Endonucleases have also been described that are specific for depurinated sites in DNA[79,80,100,132,133,134]. Endonuclease II of E. coli has been shown to introduce single-strand breaks into DNA alkylated with N-methyl-N-nitrosourea (MNU), and to release O^6-methylguanine and 3-methyl-adenine from the DNA[60]. Evidence for specific excision of certain alkylated bases from DNA treated in vivo with various monofunctional alkylating agents has been obtained in a number of systems[19,28,70,91]. The increase in single-strand molecular weight of the prelabeled DNA in NC-treated cells seen after incubation in growth medium is similar to the phenomenon observed in wild-type cells after UV irradiation[114], which was interpreted in terms of filling in of gaps formed by excision of

pyrimidine dimers. In the case of UV-irradiated cells, however, strand breaks appeared after a period of postirradiation incubation, rather than being present immediately after irradiation. Alternatively, the alkylation-induced single-strand breaks might arise from formation of apurinic sites followed by production of breaks at the apurinic sites after the cells are lysed in alkali[35, 126]. No matter what the origin of the single strand breaks are, the disappearance with time of these breaks clearly indicates some kind of repair process. At low doses of NC in H. influenzae this repair is very efficient, since almost all of the strand breaks disappeared (Figure 15, p. 51). This repair may be much faster than the data suggest, because some of the breaks may arise during the posttreatment incubation of the cells.

If the type of repair detected by the alkaline sucrose gradient experiment of Figure 15 is error-prone, it could account for part or all of the mutation fixation that occurred in the absence of DNA replication. Repair of mutagen-induced single strand breaks is probably not involved in the major, replication-dependent mutation fixation, however, because strain recl, in which all of the mutation occurs by the non-replicative pathway(s), repairs the strand breaks just as well as the wild-type strain (Figure 15). It appears from previous work[54] that repair of single-strand breaks induced by monofunctional alkylating agents does not contribute significantly to mutagenesis in H. influenzae, since MMS and MNNG produce about equal numbers of strand breaks at doses giving approximately equal survival, both kinds of strand breaks are repaired, but no mutagenesis was detected with MMS. It is possible, however, that repair of MMS-induced strand breaks occurs by a different,

less error-prone mechanism than repair of MNNG-induced breaks. In E. coli, in which MMS is highly mutagenic, repair of MMS-induced strand breaks may be very error-prone. MMS is also mutagenic in B. subtilis, and there is evidence that repair of MMS-induced strand breaks is error-prone in this organism. A strain of B. subtilis has been isolated which is deficient in the repair of MMS-induced single-strand breaks[102]. This strain is MMS-sensitive, UV-sensitive and recombination-deficient. The MMS-sensitivity mutation was transferred to a histidine-requiring strain and the ability of this strain to revert to prototrophy after treatment with MMS, UV and MNNG was tested[44]. This strain was not mutable by MMS or UV, suggesting that MMS-induced mutagenesis involves error-prone repair of strand breaks by a mechanism that is also involved in UV-induced mutagenesis. This MMS-sensitive strain was mutable by MNNG, however, suggesting that MMS-induced mutagenesis and MNNG-induced mutagenesis do not occur by the same mechanism in B. subtilis. The ability of the MMS-sensitive strain to repair MNNG-induced single-strand breaks was not reported. In summary, there is a large amount of evidence that several repair mechanisms might exist for handling alkylation-induced lesions in the DNA. Any of these could be error-prone.

Besides one or more excision-repair mechanisms that might act to remove alkylation-induced lesions from the DNA before DNA replication, a postreplication type of repair has been shown to occur in NC-treated H. influenzae (Figures 17, p. 56, and 18, p. 59) and in MMS-treated E. coli.[102] As stated before, mistakes made during postreplication repair could account for the major, replication-dependent mode of

mutation fixation. In Figure 20 a hypothetical postreplication repair process, similar to that proposed by Rupp et al.[106] for UV-irradiated E. coli, is diagrammed. When DNA containing alkylation-induced lesions (1) is replicated, discontinuities appear in the newly synthesized daughter strands opposite the lesions (2), either because incorporation cannot occur opposite certain lesions or because these lesions inhibit complete joining of Okazaki fragments. Strand exchange would result in structures in which the lesions are no longer opposite gaps. In the model of Rupp et al.[106], a structure like (3a) was proposed, but recent evidence suggests that (3b) might also be formed[36]. Filling in of the gaps by repair synthesis would lead to structure (4). If this repair synthesis were error-prone, mutation fixation would occur either in the parental (4a) or daughter (4b) strand, or both. If the repair synthesis is not extensive and most of the mutation fixation occurs in the parental strand (4a) this might explain the failure of Kimball and Setlow[58] to observe a significant increase in the sensitivity to 313-nm light of mutations newly fixed in the presence of dBrUrd, compared to mutations fixed in the absence of dBrUrd.

Concluding Comments

The work presented here has demonstrated that most of the mutagenesis induced in H. influenzae by NC involves DNA replication. It is not yet known whether in the mutagen-treated cells mispairing opposite certain alkylation-induced lesions occurs during replication, or whether errors are made during repair of interruptions in DNA strands synthesized after treatment with mutagen. Repair of mutagen-induced lesions,

Figure 20. Hypothetical scheme for error-prone joining of newly synthesized DNA strands in cells treated with N-nitroso compounds.

During replication gaps appear in the newly synthesized strands, either because the DNA polymerase fails to incorporate opposite certain mutagen-induced lesions (X) in the parental DNA, or because these lesions inhibit complete joining of Okazaki fragments. Recombination between homologous parental and daughter strands results in structures in which the gaps are no longer opposite the lesions. Error-prone repair synthesis then fills in the gaps, resulting in alterations in the nucleotide sequence in either the parental or daughter strand.

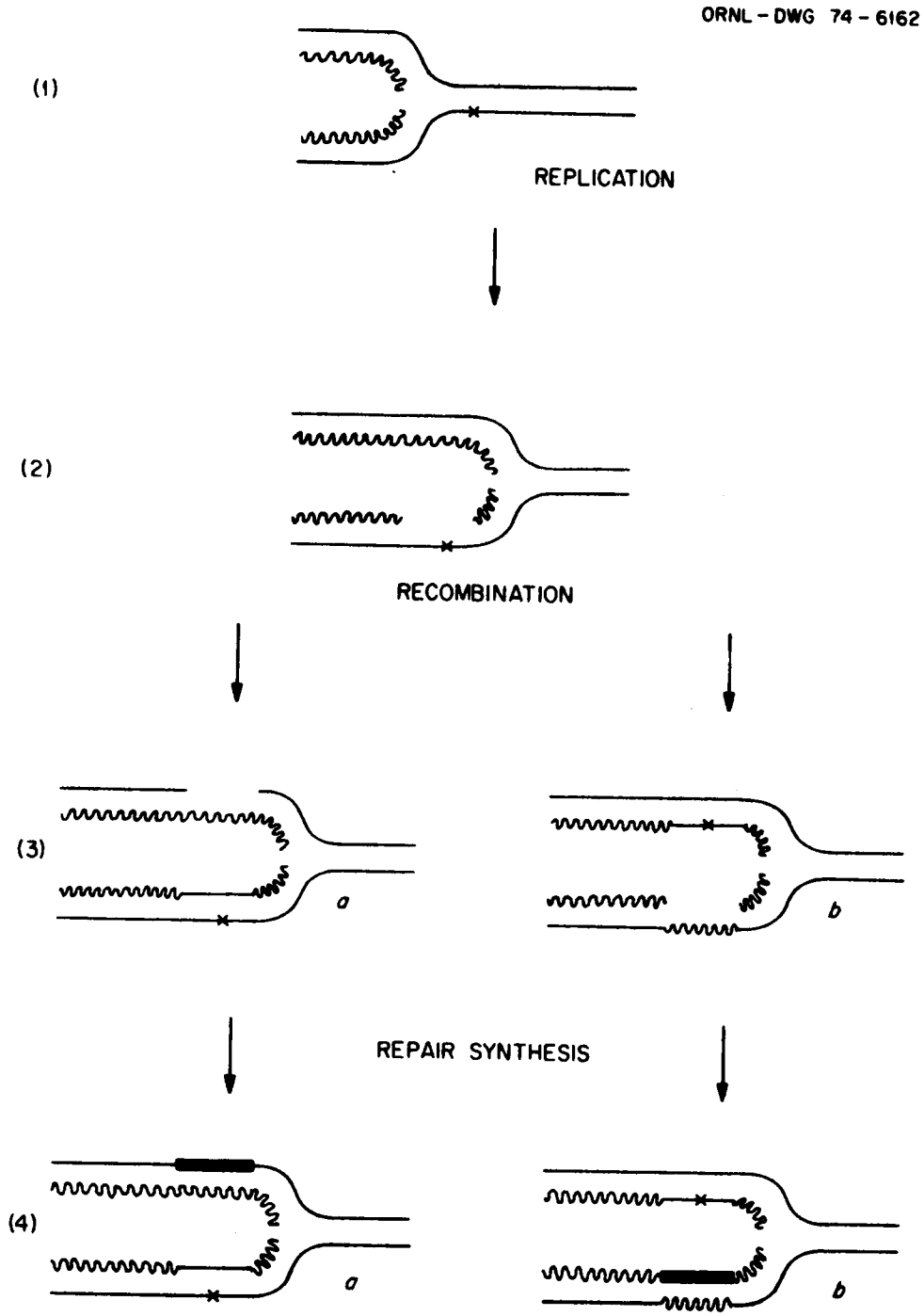

ORNL - DWG 74 - 6162
(1)
REPLICATION
(2)
RECOMBINATION
(3)
a
b
REPAIR SYNTHESIS
(4)
a
b

Figure 20

independent of DNA replication, is probably responsible for a substantial portion of the mutagenesis and is apparently also capable of removing premutational lesions before replication-associated mutation fixation occurs.

Further information concerning the cellular processes involved in repair of premutational lesions and nonreplicative mutation fixation may emerge during the examination of the large number of repair- and recombination-defective mutants available in H. influenzae. While the genetic and physical methods employed up till now have contributed significantly toward understanding the cellular processes of mutagenesis, the molecular details are more difficult to investigate, and will require in vitro enzymological studies of individual cellular functions.

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